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APPEAL BRIEF

Appellant	:	Tada, et al.
App. No	:	10/537,320
Filed	:	June 2, 2005
For	:	DENDRITE ELONGATION INHIBITOR FOR MELANOCYTE AND SKIN PREPARATION FOR EXTERNAL USE CONTAINING THE SAME
Examiner	:	Marcos Sznaidman
Art Unit	:	1628

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the Notice of Appeal filed January 3, 2011, Appellant submits this Appeal Brief.

I. REAL PARTY IN INTEREST

The real party in interest is POLA CHEMICAL INDUSTRIES, INC. POLA CHEMICAL INDUSTRIES, INC. is the assignee of record.

II. RELATED APPEALS AND INTERFERENCES

There are no related Appeals or Interferences.

III. STATUS OF CLAIMS

The following claims are rejected: 7, 12-14, 16 and 17

The following claims are allowed: none

The following claims are withdrawn: none

The following claims are canceled: 1-6, 8-11, and 15

The following claims are objected to: none

The following claims are appealed: 7, 12-14, 16 and 17

IV. STATUS OF AMENDMENTS

All amendments have been entered.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A. General Description

As skin ages, age spots, freckles and darkening begin to appear. Cosmetic products have been developed to treat this condition which work by inhibition of tyrosinase which in turn inhibits melanin production. However, not all skin discoloration is the result of overproduction of melanin. Some conditions, such as dyschromatosis, are the result of elongation of melanocytic dendrites. Products that work by inhibition of melanin production are not effective to treat age spots, freckles and darkening of skin which are caused by elongation of melanocytic dendrites (see substitute specification of 10/20/2009, page 1, last paragraph to page 2 first full paragraph).

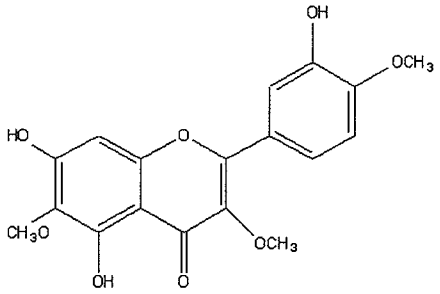
It has been found by the present inventors that Centaureidin treats skin discoloration, such as dyschromatosis, for which inhibitors of tyrosinase (melanin production) are not effective. That is, Centaureidin inhibits skin discoloration by inhibition of elongation of melanocytic dendrites. Centaureidin is effective to whiten skin for conditions in which tyrosinase inhibitors are not effective (see substitute specification of 10/20/2009, page 3, last paragraph to page 5, paragraph 2).

B. Specific Support for Independent Claims[1]

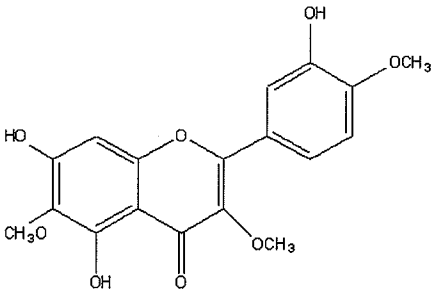
CLAIM 7

A method for whitening skin comprising: a step of applying Centaureidin represented by the following formula and/or a salt thereof to the skin of an individual in need of skin whitening,	See page 2, first full paragraph; Page 3, last paragraph; page 6, paragraphs 4-6; pages 9-10, bridging paragraph; Examples 2-4;
whereby elongation of melanocytic dendrites is inhibited:	Page 3, last paragraph; Items (2)-(5) on pages 4-5

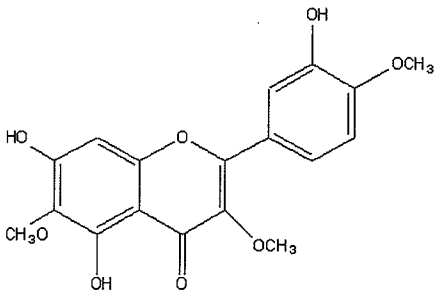
[1] Reference is to substitute specification submitted October 20, 2009

	Last paragraph, page 4, first structure on page 5

CLAIM 12

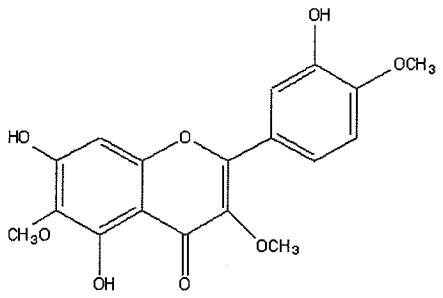
A method for whitening skin comprising applying a skin preparation for external use comprising 0.005 to 5% by weight of Centaureidin represented by the following formula and/or a salt thereof to an individual in need of skin whitening,	See page 2, first full paragraph; page 6, paragraphs 4-6; page 8, last paragraph; pages 9-10, bridging paragraph; Examples 2-4
whereby elongation of melanocytic dendrites is inhibited:	Page 3, last paragraph; Page 4, paragraph 1
	Last paragraph, page 4, first structure on page 5

CLAIM 14

A method for treating dyschromatosis comprising: a step of applying Centaureidin represented by the following formula and/or a salt thereof to the skin of an individual in need of a treatment for dyschromatosis,	See page 2, first full paragraph; Page 3, last paragraph; page 6, paragraphs 4-6; Pages 12-13, bridging paragraph; Examples 2-4;
whereby elongation of melanocytic dendrites is inhibited:	Page 3, last paragraph; Page 4, paragraph 1
	Last paragraph, page 4, first structure on page 5

CLAIM 16

A method for treating dyschromatosis comprising applying a skin preparation for external use comprising 0.005 to 5% by weight of Centaureidin represented by the following formula and/or a salt thereof to an individual in need of a treatment for dyschromatosis,	See page 2, first full paragraph; Page 3, last paragraph; page 6, paragraphs 4-6; page 8, last paragraph; Pages 12-13, bridging paragraph; Examples 2-4;
whereby elongation of melanocytic dendrites is inhibited:	Page 3, last paragraph; Page 4, paragraph 1

	Last paragraph, page 4, first structure on page 5

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 7, 12-14 and 16-17 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Ishida, et al. (EP 1147764).

VI. ARGUMENT

Claims 7, 12-14 and 16-17 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Ishida, et al. (EP 1147764).

The patient population of the claimed invention is not identical to the patient population of the cited reference.

Ishida does not teach skin whitening by inhibition of elongation of melanocytic dendrites which means that the compounds of Ishida do not work on exactly the same population as the compounds of the present invention.

Claim 7 on Appeal is directed to applying Centaureidin to "skin of an individual in need of skin whitening, whereby elongation of melanocytic dendrites is inhibited". The Examiner erred in not giving this recited limitation patentable weight. A similar limitation is found in each of claims 12, 14, and 16.

The Federal Circuit in Jansen v. Rexall Sundown, Inc. (342 F.3d 1329 (Fed. Cir. 2003); (Exhibit A; copy provided as Attachment A to the Response filed October 28, 2008) has ruled that the phrase "an individual in need thereof" must be accorded weight in determination of patentability.

Centaureidin of the present invention inhibits elongation of melanocytic dendrites (see Example 1 of the present specification) and is effective for alleviating dyschromatosis on which melanin production inhibitors utilizing a tyrosinase inhibitory action are not effective or are less effective. See Examples 2, 3 and 4 of the present specification. In contrast, Ishida, et al. teach compounds which have a whitening effect by inhibition of melanin production. Table 1 on page 8 of Ishida, et al. teaches compounds of Formulas II to V which inhibit melanin production.

Compounds of the present invention inhibit elongation of melanocytic dendrites and do not appreciably affect melanin synthesis or tyrosinase, TRP-1, TRP-2, and Pmel-17. In support, Appellants present Exhibit B (Tada, et al. IFSCC Magazine, presented at 23rd IFSCC Congress 2004, Orlando FL; Attachment B to the Response filed October 28, 2008). The paper reports on the effects of a number of botanical extracts on melanosomes and melanocytic dendrites, including centaureidin. While centaureidin was effective to shrink normal human melanocyte

dendrites (Figure 2) and to inhibit melanosome transfer through dendrites (Figure 5), effects on melanin production were negligible (Figure 4).

Accordingly, a person in need of a skin treatment "whereby elongation of melanocytic dendrites is inhibited" would not turn to the compounds of the disclosure of Ishida, et al. because there is no indication in Ishida, et al. that the compounds disclosed by Ishida, et al. are useful to inhibit melanocytic dendrite elongation. Furthermore, the 1st Tada Declaration shows that a compound (Nobiletin) according to Ishida, indeed does not inhibit dendrite length.

First TADA Declaration under 37 C.F.R. § 1.132

The 1st Tada Declaration (Exhibit C, submitted on April 22, 2009 in response to the Final Office Action of January 27, 2009) compares Nobiletin, corresponding to Formula (III) of Ishida which corresponds to general Formula (I) of Ishida, et al. to Centaureidin of the presently claimed invention.

As can be seen from item 7 in the TADA Declaration, the formulation corresponding to Ishida, et al. (Nobiletin) does not inhibit dendrite length. Measurements are comparable to the control. In contrast, Centaureidin significantly inhibits dendrite length (30 +/-10 μm).

Clearly the claims on appeal are directed to the treatment of a different condition than disclosed by Ishida, et al. Ishida, et al. is directed to treatment of conditions caused by melanin overproduction. As discussed in the Background section of the present specification, some forms of dyschromatosis cannot be effectively treated by inhibitors of melanin production (see substitute specification of 10/20/09, page 1, last line to page 2, line 12). Appellants' claimed invention is directed to treatment of conditions caused by elongation of melanocytic dendrites. While both treatments relate to skin, the two treatments are not interchangeable as some skin conditions result from elongation of melanocytic dendrites rather than melanin production.

The compounds of Ishida, et al. have an effect opposite to the effects of centaureidin. Namely, the compounds of Ishida, et al. inhibit melanin production while centaureidin has negligible effect on melanin production (Figure 4 of Exhibit B, discussed above).

Accordingly, the claimed method directed to Centaureidin, is non-obvious over the methods taught with the compounds of Ishida, et al. which have a different action than Centaureidin. The compounds of Ishida, et al. inhibit melanin production but do not inhibit

dendrite elongation as shown by the TADA Declaration (Exhibit C). Centaureidin of the claimed invention inhibits dendrite elongation but does not have appreciable effect on melanin production as shown by the TADA reference (Exhibit B). Accordingly, the person in need of application of Centaureidin is different from the person in need of application of the compounds of Ishida.

Application of the method of Ishida, et al, in which inhibitors of melanin production are applied to skin, would not be satisfactory for treatment of forms of dyschromatosis in which inhibitors of melanin production are not satisfactorily effective. An "individual in need" of a skin treatment to inhibit elongation of melanocytic dendrites would not be effectively treated with the compounds disclosed by Ishida, et al.

Ishida, et al. do not teach Centaureidin, the compound recited in the treatment method of the claimed invention.

Ishida, et al. do not teach Centaureidin, the compound recited in the methods of the presently claimed invention. Ishida, et al. disclose a general formula (1) (page 3) which is generic to formula (1) of Appellants' claim 1, but there is no teaching or suggestion that any of the compounds falling within the genus of formula (1) of Ishida, et al. are effective to inhibit melanocytic dendrite elongation. Ishida, et al. only teach inhibition of melanin production.

The compounds of Ishida, et al., while structurally related to the compounds of Appellants, have an effect opposite to the effects of centaureidin. Namely, the compounds of Ishida, et al. inhibit melanin production while centaureidin has negligible effect on melanin production as discussed above with respect to Tada, et al. (Exhibit B).

The Final Office Action of 1/7/2010 and Office Action of 10/4/2010 point out that the claimed compound (Centaureidin) differs from Ishida, et al. in having 3 methoxy groups while Ishida, et al. teach "at least four methoxy groups" (Final Office Action of 1/7/2010, page 6, paragraph 2; Office Action of 10/4/2010 at page 5, 4th and 5th line from bottom). The 1/7/2010 and 10/4/2010 Office Actions state that "it is expected that compounds of general structure I taught by Ishida et al. and Centaureidin of the instant application, differing by only one -CH3 group, would have similar chemical physical and biochemical properties" (Final Office Action of 1/7/2010, page 7, end of 1st paragraph; also page 8, paragraph 3; also page 11, end of paragraph 1; see also Office Action of 10/4/2010, page 6, 1st paragraph).

Despite this structural similarity, Centaureidin has a very different action compared to the compounds of Ishida. As shown in Table 1 of Ishida, et al. on page 8, some of the compounds of Ishida, et al., including Compound III which is used in Examples 1, 3-5, and 7-11 of Ishida, et al., inhibit melanin production. However, Ishida, et al. does not teach any compound that inhibits length of dendrites. As discussed above, Centaureidin of the claimed invention has the advantageous action of inhibiting elongation of melanocytic dendrites, thereby treating skin discoloration that cannot be treated by the compounds of Ishida, et al.

As shown in the previously submitted 1st Tada Declaration (Exhibit C), Nobiletin, which corresponds to Compound III of Ishida, et al. and which is used in Examples 1, 3-5, and 7-11 of Ishida, et al., has no significant effect on dendrite length over a concentration range of 10^{-6} % to 10^{-4} %. In contrast, centaureidin, according to the claimed invention, has a dramatic inhibitory effect on dendrite length. In view of the structural similarity between centaureidin according to the claimed invention and compound III of Ishida, et al., this dramatically different effect was unexpected. This is directly contrary to the expectations expressed in the Office Actions that compounds of similar structure, would have similar chemical physical and biochemical properties. The Office Actions at least implicitly admits that the effects demonstrated by the claimed invention were unexpected.

The Office Actions of 1/7/2010 and 10/4/2010 state that "products of identical or similar composition cannot exert mutually exclusive properties when administered under the same circumstance" (Final Office Action of 1/7/2010, page 8, paragraph 2; also page 12, paragraph 2; see also Office Action of 10/4/2010 at page 7, first full paragraph). In response, neither Centaureidin (according to the claimed invention) nor Nobiletin (according to Ishida) are capable of whitening skin of all types. Centaureidin is effective to inhibit dendrite lengthening, but not particularly effective to inhibit melanin production (Exhibits B and C discussed above) while Nobiletin is not effective to inhibit dendrite elongation but is effective in inhibition of melanin production (see Table 1 of Ishida and Tada Declaration).

The Final Office Action of 1/7/2010 and Office Action of 10/4/2010 state that "the discovery of a previously unappreciated property of a prior art composition or a scientific explanation for the prior art's functioning does not render the old composition patentably new to the discoverer (Final Office Action of 1/7/2010, page 8, paragraph 2, citing M.P.E.P. 2112; also

page 12, paragraph 2; see also Office Action of 10/4/2010, page 7, first full paragraph). However, such is not the case here. Centaureidin is ***chemically distinct*** from the compounds taught by Ishida, et al. and has ***different properties and function***.

Centaureidin has the remarkable effect of inhibiting dendrite length. This effect is important for a subject whose pigmentation is caused not only by production of melanin, but also by elongation of melanocytic dendrites. The compounds of Ishida, et al. are not effective to inhibit dendrite length and so would have minimal effect to whiten skin in the case where pigmentation is caused by elongation of melanocytic dendrites.

The Examiner erred in discounting the phrase "whereby elongation of melanocytic dendrites is inhibited" as merely the intended result of the process steps positively recited (Final Office Action of 1/7/2010, page 7, last paragraph; also page 11, last paragraph; see also Office Action of 10/4/2010, page 6, last paragraph). However, in M.P.E.P. 2111.04 cited by the Examiner it also states that "when a 'whereby' clause states a condition that is material to patentability; it cannot be ignored in order to change the substance of the invention". In the present case, the cited reference, Ishida, et al. do <u>not</u> show inhibition of elongation of melanocytic dendrites as shown by the 1st Tada Declaration. It is improper to ignore the evidence presented by Appellants. Centaureidin is a chemically different compound that acts in a different way to whiten skin compared to the teaching of Ishida, et al. Accordingly, if dark complexion is caused by both melanin production and elongation of melanocytic dendrites, then application of centaureidin according to the claimed invention will further whiten skin even after treatment with compounds according to Ishida, et al. The "substance of the invention" is that the claimed method of Appellants is effective even under circumstances when the compounds of Ishida, et al. are ineffective.

In particular, claims 14, 16 and 17 relate to treatment of dyschromatosis. The compounds of Ishida, et al. will have minimal effect on dyschromatosis as the compounds of Ishida, et al. do not inhibit dendrite length as demonstrated by the 132 Declaration of Akihiro Tada (Exhibit C), discussed above.

The Office Action states that "the prior art establishes that both [centaureidin and compounds of Ishida, et al] function in similar manner" (Final Office Action of 1/7/2010, page 12, last paragraph; Office Action of 10/4/2010, page 7, last paragraph). However, as discussed

above, the compounds of Ishida, et al. and the claimed compound, centaureidin, function in a completely different manner such that one may be effective where another is not or the use of the two together may be more effective than the use of either one by itself. The two compounds are not interchangeable with respect to effect.

Second Tada Declaration

Centaureidin of the claimed method has a different result on the same patient population as Nobiletin (the compound of Ishida) as demonstrated by the 2nd Tada Declaration (submitted with the Response of June 28, 2010, Exhibit D).

Melanin granules are produced by melanocytes in the basal epidermal layer. The melanin granules produced by the melanocyte are transferred to keratinocytes by elongation of melanocyte dendrites.

Nobiletin has an action of inhibiting production of melanin granules by inhibiting tyrosinase. However, as indicated in the 1st Tada Declaration (Exhibit C), Nobiletin does not inhibit elongation of the dendrite.

Consequently, Nobiletin does not inhibit the transfer of melanin granules to keratinocytes when tyrosinase in melanocytes has been activated by exposure to UV and the like, or when tyrosinase in melanocytes is activated congenitally. Thus, Nobiletin does not sufficiently inhibit pigmented spots or dark complexion.

On the other hand, Centaureidin inhibits elongation of dendrites of melanocytes. Therefore, Centaureidin inhibits the transfer of melanin granules to keratinocytes even after tyrosinase in melanocytes has been activated by exposure to UV or the like, or when tyrosinase in melanocytes is activated congenitally. Thus, Centaureidin effectively inhibits pigmented spot and dark complexion.

Centaureidin does not inhibit melanin production as shown in the 2nd Tada Declaration (Exhibit D).

Furthermore, the effective concentration of Centaureidin required to inhibit the elongation of melanocytic dendrites is much less than the effective concentration of Nobiletin to inhibit the production of melanin granules.

Referring to the 1st Tada Declaration (Exhibit C) the Declaration shows that 10^{-5} % Centaureidin is effective to inhibit the elongation of melanocytic dendrites. 10^{-5} % of Centaureidin corresponds to about 0.27 μM.

On the other hand, as show in the 2nd Tada Declaration (Exhibit D), the effective concentration of Nobiletin to inhibit the production of melanin granules is more than 1 μM.

As discussed above, the effects of Centaureidin and Nobiletin (as taught by Ishida, et al.) are clearly different from each other. Although Centaureidin and Nobiletin are structurally similar to each other, the effectiveness observed with Centaureidin in inhibition of pigmented spot or dark complexion is not obtained using Nobiletin. This result cannot be derived from Ishida, et al. and is unexpected.

The claims on appeal are directed to treatment of different conditions that taught by Ishida

Ishida is directed to treatment of conditions caused by melanin overproduction. Appellants' claimed invention is directed to treatment of conditions caused by elongation of melanocytic dendrites. While both treatments relate to skin, the two treatments are not interchangeable as some skin conditions result from elongation of melanocytic dendrites rather than melanin production. Accordingly, the reasons for the similar symptoms are different and require different treatment.

Conclusion

In view of the foregoing arguments distinguishing Claims 7, 12-14 and 16-17 over the prior art, Appellant respectfully requests that the rejection of these claims be reversed.

Please charge any additional fees, including any fees for additional extensions of time, or credit overpayment to Deposit Account No. 11-1410.

Dated: February 17, 2011

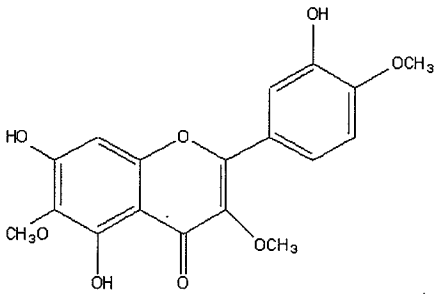
By:/Che S. Chereskin/
Che Swyden Chereskin
Registration No. 41,466
Agent of Record
Customer No. 20995
(949) 721-6385

VII. CLAIMS APPENDIX

1-6. (Canceled)

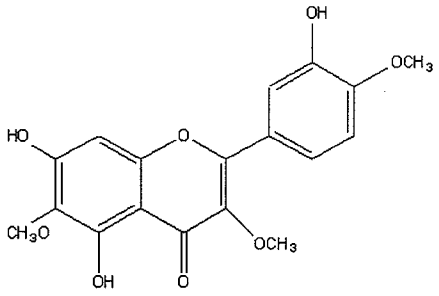
7. A method for whitening skin comprising:

a step of applying Centaureidin represented by the following formula and/or a salt thereof to the skin of an individual in need of skin whitening, whereby elongation of melanocytic dendrites is inhibited:



8-11. (Cancelled)

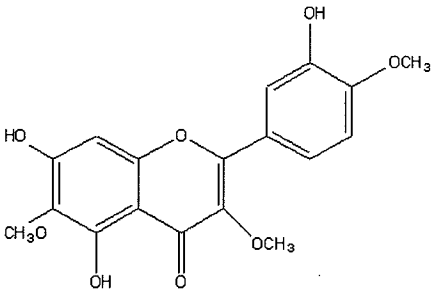
12. A method for whitening skin comprising applying a skin preparation for external use comprising 0.005 to 5% by weight of Centaureidin represented by the following formula and/or a salt thereof to an individual in need of skin whitening, whereby elongation of melanocytic dendrites is inhibited:



13. The method for whitening skin according to claim 12, wherein the skin preparation for external use is a cosmetic.

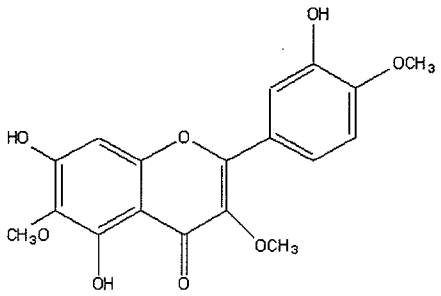
14. A method for treating dyschromatosis comprising:

a step of applying Centaureidin represented by the following formula and/or a salt thereof to the skin of an individual in need of a treatment for dyschromatosis, whereby elongation of melanocytic dendrites is inhibited:



15. (Canceled)

16. A method for treating dyschromatosis comprising applying a skin preparation for external use comprising 0.005 to 5% by weight of Centaureidin represented by the following formula and/or a salt thereof to an individual in need of a treatment for dyschromatosis, whereby elongation of melanocytic dendrites is inhibited:



17. The method for treating dyschromatosis according to claim 16, wherein the skin preparation for external use is a cosmetic.

VIII. EVIDENCE APPENDIX

Exhibit A: Jansen v. Rexall Sundown, Inc. (342 F.3d 1329 (Fed. Cir. 2003); (copy provided as Attachment A to the Response filed October 28, 2008).

Exhibit B: Tada, et al. IFSCC Magazine, presented at 23rd IFSCC Congress 2004, Orlando FL (Attachment B to the Response filed October 28, 2008).

Exhibit C: 1st Tada Declaration (submitted on April 22, 2009 in response to the Final Office Action of January 27, 2009).

Exhibit D: 2nd Tada Declaration (submitted with the Response of June 28, 2010)

Exhibit E: Definition from online source which defines "dyschromatosis" as "an asymptomatic anomaly of pigmentation occurring among the Japanese; may be localized or diffuse"(Attachment to Amendment filed October 19, 2009).

Exhibit F: Ishida, et al. EP 1147764A2 (IDS of June 2, 2005).

IX. RELATED PROCEEDINGS APPENDIX

There are no related proceedings.

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EXHIBIT A

342 F3d 1329 (Sept. 8, 2003)

United States Court of Appeals for the Federal Circuit

03-1069

CHRISTIAN J. JANSEN, JR.,

Plaintiff-Appellant,

v.

REXALL SUNDOWN, INC.,

Defendant-Appellee.

John C. McNett, Woodard, Emhardt, Naughton, Moriarty & McNett, of Indianapolis, Indiana, argued for plaintiff-appellant. With him on the brief was Steve E. Zlatos.

Gary H. Levin, Woodcock Washburn LLP, of Philadelphia, Pennsylvania, argued for defendant-appellee. With him on the brief was Lynn B. Morreale.

Appealed from: United States District Court for the Southern District of Indiana

Judge John Daniel Tinder

United States Court of Appeals for the Federal Circuit

03-1069

CHRISTIAN J. JANSEN, JR.,

Plaintiff-Appellant,

v.

REXALL SUNDOWN, INC.,

Defendant-Appellee.

DECIDED: September 8, 2003

Before LOURIE, RADER, and SCHALL, Circuit Judges.

LOURIE, Circuit Judge.

Christian J. Jansen, Jr., appeals from the final decision of the United States District Court for the Southern District of Indiana granting summary judgment that Rexall Sundown, Inc. has not infringed Jansen's U.S. Patent 4,945,083. Jansen v. Rexall Sundown, Inc., No. IP 00-1495-C-T/G (S.D. Ind. Sept. 25, 2002). Because the court correctly construed the patent claims and correctly found no genuine issues of material fact on the question of infringement, we affirm.

BACKGROUND

Jansen is the sole inventor and owner of the '083 patent, which is directed to methods of "treating or preventing macrocytic-megaloblastic anemia" by administering a combination of folic acid and vitamin B_{12} "to a human in need thereof." '083 patent, col. 6, ll. 20-24, ll. 37-41. According to the patent, deficiencies of either folic acid or vitamin B_{12} can cause macrocytic-megaloblastic anemia, also referred to as pernicious anemia, while

a deficiency of vitamin B_{12} can also cause neurological problems. Id. at col. 4, ll. 13-25. When folic acid alone is utilized to treat macrocytic-megaloblastic anemia, the folic acid may mask a vitamin B_{12} deficiency. Id.; see also id. at col. 3, l. 65 – col. 4, l. 5. An objective of Jansen's invention is to administer both supplements together to avoid the masking problem. Id. at col. 4, ll. 25-48. The independent claims read as follows:

> 1. A method of treating or preventing macrocytic-megaloblastic anemia in humans which anemia is caused by either folic acid deficiency or by vitamin B_{12} deficiency which comprises administering a daily oral dosage of a vitamin preparation to a human in need thereof comprising at least about 0.5 mg. of vitamin B_{12} and at least about 0.5 mg. of folic acid.
>
> 4. A method of treating or preventing macrocytic-magaloblastic [sic] anemia in humans which anemia is caused by either folic acid deficiency or by vitamin B_{12} deficiency which comprises orally administering combined vitamin B_{12} and folic acid to a human in need thereof in sufficient amounts to achieve an oral administration of at least about 0.5 mg. of vitamin B_{12} and at least about 0.5 mg. of folic acid within one day.

Id. at col. 6, ll. 20-24, ll. 37-41 (emphases added).

The '083 patent is a seventh-generation continuation of a patent application filed in 1970. Every member of the '083 patent's lineage was abandoned in favor of the succeeding application until the '083 patent issued in 1990. Jansen's first application claimed the method as follows:

> A method of treating or preventing anemia in humans which comprises administering a daily oral dosage of a vitamin preparation containing at least .5 mg. of vitamin B_{12} and at least .5 mg. of folic acid, whereby anemia can safely be treated orally without determining whether it is caused by folic acid deficiency or by vitamin B_{12} deficiency.

In re Jansen, 187 USPQ 743, 744 (CCPA 1975). That original claim, while specifying approximately the same amounts of folic acid and vitamin B_{12}, does not specify the type of anemia being treated and says nothing about any need on the part of the human subject. The U.S. Patent and Trademark Office ("PTO") found that claim, as well as claims directed to the composition of matter, to be obvious in light of prior art that taught administration of folic acid alone in the claimed range, vitamin B_{12} alone in the claimed range, and

combinations of the two in smaller doses than claimed. The PTO found unpersuasive Jansen's argument that administration of both components in the higher, claimed doses was an unexpected solution to the masking problem, and the Court of Customs and Patent Appeals affirmed the PTO's rejections. Id. at 746. In his next five applications, Jansen persistently attempted to gain allowance of his claims in slightly different form, yet the PTO consistently rejected his attempts. In the prosecution of his seventh application, Jansen repeated his masking-avoidance argument and submitted an article that asserted that the medical community had come to realize the effectiveness of folic acid-vitamin B_{12} combination therapy to treat pernicious anemia only after Jansen's invention date. See William H. Crosby, Improvisation Revisited — Oral Cyanocobalamin Without Intrinsic Factor for Pernicious Anemia, 140 Arch. Intern. Med. 1582 (1980). The examiner agreed but noted that the claims, being directed to unspecified anemia, were not commensurate in scope with Jansen's showing of unexpected results. Jansen thereafter agreed to cancel his composition of matter claims and to narrow his method claims by requiring a specific type of anemia, viz., macrocytic-megaloblastic anemia, rather than anemia generally, and by adding to the claims the phrase "to a human in need thereof." The PTO then issued the '083 patent to Jansen.

Rexall markets to the general public an over-the-counter dietary supplement presently known as Folic Acid XTRA™ that contains folic acid and vitamin B_{12} within the claimed ranges. The Rexall product is labeled and advertised for maintenance of proper blood homocysteine levels, but not for prevention or treatment of macrocytic-megaloblastic anemia.

Jansen sued Rexall for inducement of and contributory infringement of the '083 patent. In the district court Jansen argued that all people are "human[s] in need" of "treat[ment] or prevent[ion] of macrocytic-megaloblastic anemia," but the court, without

definitively construing the "in need" phrase, rejected that argument. Jansen, slip op. at 14. Citing, inter alia, Rapoport v. Dement, 254 F.3d 1053 (Fed. Cir. 2001), the court then construed the phrase "treating or preventing macrocytic-megaloblastic anemia" to require that, in order to infringe the patent, the human subject of the claimed method take the compound with the intent of treating or preventing macrocytic-megaloblastic anemia. Jansen, slip op. at 16. Because the court found no evidence of such intent or purpose on the part of Rexall's customers, the court granted summary judgment of noninfringement. Id. at 16-17.

Jansen timely appealed to this court, and we have jurisdiction pursuant to 28 U.S.C. § 1295(a)(1).

DISCUSSION

Summary judgment is appropriate if "there is no genuine issue as to any material fact and . . . the moving party is entitled to a judgment as a matter of law." Fed. R. Civ. P. 56(c). "The evidence of the nonmovant is to be believed, and all justifiable inferences are to be drawn in his favor." Anderson v. Liberty Lobby, Inc., 477 U.S. 242, 255 (1986). We review a district court's grant of a motion for summary judgment de novo. Ethicon Endo-Surgery, Inc. v. U.S. Surgical Corp., 149 F.3d 1309, 1315 (Fed. Cir. 1998).

A determination of patent infringement requires a two-step analysis. "First, the court determines the scope and meaning of the patent claims asserted . . . [Second,] the properly construed claims are compared to the allegedly infringing device." Cybor Corp. v. FAS Techs., Inc., 138 F.3d 1448, 1454 (Fed. Cir. 1998) (en banc) (citations omitted). Step one, claim construction, is an issue of law, Markman v. Westview Instruments, Inc., 52 F.3d 967, 970-71 (Fed. Cir. 1995) (en banc), aff'd, 517 U.S. 370 (1996), that we review de novo, Cybor, 138 F.3d at 1456. Step two, comparison of the claim to the accused device, requires a determination that every claim limitation or its equivalent is found in the

accused device. Warner-Jenkinson Co. v. Hilton Davis Chem. Co., 520 U.S. 17, 29 (1997). Those determinations are questions of fact. Bai v. L & L Wings, Inc., 160 F.3d 1350, 1353 (Fed. Cir. 1998).

On appeal, Jansen first argues that the court improperly construed the claims. More specifically, he contends that the court's construction improperly added to the claims an intent element, which is contrary to law as well as contrary to the ordinary meaning of the claim language, which does not suggest that the infringer's state of mind is relevant. Nor does the '083 patent's prosecution history, according to Jansen, suggest that the infringer's state of mind is relevant. He also argues that Rapoport does not support the court's view that a direct infringer must purposefully perform the claimed method, and that in any event Rapoport is distinguishable because that case, unlike this case, did not involve a claim to a method of prevention of a disease. According to Jansen, the phrase "a human in need thereof" encompasses a person who does not know that his or her serum levels of folic acid and vitamin B_{12} are adequate. Jansen secondly argues that he presented sufficient evidence of infringement to avoid summary judgment. According to Jansen, Rexall's formulation and labeling are circumstantial evidence of direct infringement by Rexall's customers.

Rexall responds that the court's claim construction does not add an intent element to the claims except as required by the particular language of the claims themselves. Rexall also contends that, just as in Rapoport, the claims in the '083 patent should be interpreted to require that the target group ("human[s] in need thereof") practice the method for the stated purpose ("treating or preventing macrocytic-megaloblastic anemia"), especially where, as here, the prosecution history reveals that both limitations were added for patentability. According to Rexall, a "human in need thereof" is someone either suffering from macrocytic-megaloblastic anemia or at a recognized risk, such as by

medical diagnosis, of developing that condition. Rexall also responds that there is no evidence that it markets its product to the target group for the claimed purpose; on the contrary, it contends that it markets its product only for regulation of blood homocysteine levels. Rexall further contends that, even if there were some evidence of direct infringement by its customers, it is not liable for indirect infringement, for it has not intended to cause infringement and there are substantial noninfringing uses of its product, thereby negating inducement of and contributory infringement.

We begin our claim construction, as always, with the ordinary meaning of the claim language. Rexnord Corp. v. Laitram Corp., 274 F.3d 1336, 1341 (Fed. Cir. 2001). That language requires that the method be performed on "a human in need thereof" and that the method be used "for treating or preventing macrocytic-megaloblastic anemia." The parties do not dispute what "macrocytic-megaloblastic anemia" means; instead, they dispute how the "treating or preventing" phrase and the "to a human in need thereof" phrase should be read. The issue reduces to whether such a human must know that he is in need of either treatment or prevention of that condition.

A similar issue arose in Rapoport, an interference proceeding before the PTO's Board of Patent Appeals and Interferences. The count in that case read as follows:

> A method for treatment of sleep apneas comprising administration of a therapeutically effective amount of a Formula I azapirone compound or a pharmaceutically effective acid addition salt thereof to a patient in need of such treatment . . . .

254 F.3d at 1056 (emphases added). On appeal we gave weight to the ordinary meaning of the preamble phrase "for treatment of sleep apneas," interpreting it to refer to sleep apnea, per se, not just "symptoms associated with sleep apnea." Id. at 1059. Rapoport argued that the count was unpatentable on the ground that a prior art reference disclosed that a form of the compound recited in the claim could be administered, not for treatment of sleep apnea itself, but for treatment of anxiety and breathing difficulty, a symptom of

apnea. Id. at 1061. We rejected that argument, stating, "There is no disclosure in the [prior art reference that the compound] is administered to patients suffering from sleep apnea with the intent to cure the underlying condition." Id. (emphasis added). Thus, the claim was interpreted to require that the method be practiced with the intent to achieve the objective stated in the preamble.

Just as in Rapoport, it is natural to interpret the nearly parallel language in the '083 patent claims in the same way. In both Rapoport and this case, the claim preamble sets forth the objective of the method, and the body of the claim directs that the method be performed on someone "in need." In both cases, the claims' recitation of a patient or a human "in need" gives life and meaning to the preambles' statement of purpose. See Kropa v. Robie, 187 F.2d 150, 152 (CCPA 1951) (stating the rule that a preamble is treated as a limitation if it gives "life and meaning" to the claim). The preamble is therefore not merely a statement of effect that may or may not be desired or appreciated. Rather, it is a statement of the intentional purpose for which the method must be performed. We need not decide whether we would reach the same conclusion if either of the "treating or preventing" phrase or the "to a human in need thereof" phrase was not a part of the claim; together, however, they compel the claim construction arrived at by both the district court and this court.

Our conclusion as to the meaning of the claims is bolstered by an analysis of the prosecution history. The prosecution history is often useful to ascertain the meaning of the claim language. Indeed, claims are not construed in a vacuum, but rather in the context of the intrinsic evidence, viz., the other claims, the specification, and the prosecution history. See DeMarini Sports, Inc. v. Worth, Inc., 239 F.3d 1314, 1327 (Fed. Cir. 2001). In this case, the "treating or preventing macrocytic-megaloblastic anemia" phrase and the "to a human in need thereof" phrase were added to gain allowance of the

claims after almost twenty years of repeatedly unsuccessful attempts to gain allowance of claims without those phrases. We must therefore give them weight, for the patentability of the claims hinged upon their presence in the claim language. See Smith v. Magic City Kennel Club, Inc., 282 U.S. 784, 790 (1931) ("The applicant[,] having limited his claim by amendment and accepted a patent, brings himself within the rules that if the claim to a combination be restricted to specified elements, all must be regarded as material, and that limitations imposed by the inventor, especially such as were introduced into an application after it had been persistently rejected, must be strictly construed against the inventor and looked upon as disclaimers."). Furthermore, because both phrases were added simultaneously to overcome the same rejection, they should be read together, meaning that the word "thereof" in the phrase "to a human in need thereof" should be construed to refer to the treatment or prevention of macrocytic-megaloblastic anemia. Finally, that "need" must be recognized and appreciated, for otherwise the added phrases do not carry the meaning that the circumstances of their addition suggest that they carry. In other words, administering the claimed vitamins in the claimed doses for some purpose other than treating or preventing macrocytic-megaloblastic anemia is not practicing the claimed method, because Jansen limited his claims to treatment or prevention of that particular condition in those who need such treatment or prevention. Thus, the '083 patent claims are properly interpreted to mean that the combination of folic acid and vitamin B_{12} must be administered to a human with a recognized need to treat or prevent macrocytic-megaloblastic anemia.

Given that claim construction, we turn to the issue whether Jansen has raised a genuine issue of material fact regarding infringement. We conclude that he has not. Jansen has asserted indirect infringement by Rexall, premised on direct infringement by Rexall's customers. See Met-Coil Sys. Corp. v. Korners Unlimited, Inc., 803 F.2d 684, 687

(Fed. Cir. 1986) ("Absent direct infringement of the patent claims, there can be neither contributory infringement nor inducement of infringement." (citations omitted)). Jansen's theory of infringement is primarily based upon his construction of the claim that those who do not affirmatively know that they do not need to take steps to prevent or treat macrocytic-megaloblastic anemia are still "in need thereof." As explained above, that claim construction is incorrect. Jansen nonetheless asserts that he has circumstantial evidence of direct infringement by Rexall's customers under the claim construction we and the district court have adopted. Specifically, he contends that Rexall's formulation, having folic acid and vitamin B_{12} in such large quantities as his claims call for, as well as Rexall's labeling stating that "[i]t is especially important to take B-12 along with Folic acid because Folic acid can mask a B-12 deficiency," are evidence that some customers do knowingly take the Rexall product to treat or prevent macrocytic-megaloblastic anemia.

While Jansen is correct that it is theoretically possible that some of Rexall's customers do take the Rexall product knowingly to treat or prevent macrocytic-megaloblastic anemia, and therefore directly infringe his patent, his evidence is quite weak. In fact, he has shown no more than a theoretical possibility or "metaphysical doubt," which is insufficient to create a genuine issue of material fact. See Anderson, 477 U.S. at 261 (citing Matsushita Elec. Indus. Co. v. Zenith Radio Corp., 475 U.S. 574, 586 (1986)). The district court's decision that there were no genuine issues of material fact on the question of infringement was therefore correct.

Use of an over-the-counter product like Rexall's is quite different from the use of a product pursuant to a prescription from a medical doctor. In the latter case, a prescription is evidence of a diagnosis and a knowing need to use the product for the stated purpose. Jansen does not have evidence of that in this case. Rexall's product is provided with a label stating that the product can be used for maintenance of blood homocysteine levels,

and purchasers do not necessarily know that they are in need of preventing or treating macrocytic-megaloblastic anemia. Instead, Jansen has only conjecture that some purchasers of the Rexall product might meet the claim requirements. The district court therefore did not err in holding that he failed to present sufficient proof of infringement to create a genuine issue of material fact and to thereby avoid summary judgment of noninfringement.

CONCLUSION

The district court correctly construed the claims of the '083 patent and properly determined that Jansen did not present sufficient evidence to create a genuine issue of material fact relating to infringement by Rexall. Accordingly, we

AFFIRM.

EXHIBIT B

Control of Melanosome Transfer by Promoting Shrinkage or Expansion of Melanocyte Dendrites

Akihiro Tada, Akiko Kanamaru, Yuko Ito

POLA Laboratories, POLA Chemical Industries, Inc., 560 Kashio-cho, Totsuka-ku, Yokohama 244-0812, Japan
Corresponding author – email: akihiro@pola.co.jp

Abstract

Melanosomes synthesized within melanocytes are transferred to keratinocytes through melanocyte dendrites, resulting in a constant supply of melanin to the epidermis which determines skin pigmentation. Theoretically, if we can find an effective way to control this supply of melanin to the epidermis, skin color could be darkened or lightened. The objective of this study was to find safe and effective methods to inhibit or promote melanosome transfer by the shrinkage or expansion of melanocyte dendrites.

Methylophiopogonanone B and centaureidin inhibited melanosome transfer to keratinocytes as well as melanocyte dendrite outgrowth. Methylswertianin and comfrey extract promoted not only melanosome transfer to keratinocytes but also expansion of melanocyte dendrites.

Methylophiopogonanone B and centaureidin suppressed pigmentation in a three-dimensional skin culture model through the inhibition of melanocyte dendrite outgrowth. Methylswertianin and comfrey extract activated pigmentation in a three-dimensional skin culture model by expansion of melanocyte dendrites.

Our experimental findings suggest the possibility of manipulating human skin color by controlling melanosome transfer to cause shrinkage or expansion of dendrites. A combination of effective agents, in addition to the ones identified in this work, could result in the creation of very unique cosmetic products that would precisely control the darkening or lightening of skin tone.

Keywords: Methylophiopogonanone B, centaureidin, methylswertianin, comfrey extract, melanocyte dendrites

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INTRODUCTION

The popularity of self-tanning products in many Western nations and whitening products in Asian countries is evidence of the persistent human desire to control skin color. However, there are many difficulties with the products currently available for such purposes. For example, dihydroxyacetone-based tanning products do not always produce the desired color [1], and whitening ingredients, including many tyrosinase inhibitors such as hydroquinone and kojic acid, are ineffective at low concentrations and unsafe at high concentrations [2-4]. Thus, there is a need for better skin tone-altering products. The epidermal melanin unit consists of the symbiotic interaction between melanocytes and an associated pool of keratinocytes. It has been estimated that a single melanocyte interacts with approximately 36 keratinocytes in the basal and suprabasal layers of the epidermis [5]. Melanosomes synthesized within melanocytes are transferred to keratinocytes through melanocyte dendrites. The dendrites of melanocytes transfer melanin to surrounding keratinocytes in response to hormones such as melanocyte stimulating hormone and ultraviolet light, both of which stimulate melanin synthesis and melanosome transfer [6,7]. During this process dendrite extension is necessary for melanosome transfer to keratinocytes because melanocytes constitute a minor population in the epidermis and must supply melanin to many keratinocytes [8]. This results in a constant supply of melanin reaching the epidermis. In this way, skin pigmentation is determined. Theoretically, skin color could be darkened or lightened by controlling the supply of melanin reaching the epidermis. One possible way of controlling this supply is to promote shrinkage or expansion of dendrites. The objective of this study was to find safe and effective methods that would inhibit or promote melanosome transfer by shrinking or expanding the melanocyte dendrites. After examining many different agents for this purpose, we focused our investigation on botanical extracts since they are known to contain a large number of chemicals and to have a relatively low cytotoxicity. Thus, by screening a large number of selected extracts, promising active ingredients that would be safe and effective for our purpose could be identified.

EXPERIMENTAL

Cells and cell culture

Normal human epidermal melanocytes and normal human epidermal keratino-

cytes were obtained from Kurabo Biomedical Business (Osaka, Japan). Normal human epidermal melanocytes were main-tained in melanocyte complete 154S culture medium supplemented with 0.5% fetal bovine serum, 5 µg/mL insulin, 0.5 µM hydrocortisone, 5 µg/mL transferrin, 3 µg/mL heparin, 3 ng/mL human basic fibroblast growth factor, 10 ng/mL phorbol-12-myristate-13-acetate and 0.2% bovine pituitary extract (all purchased from Kurabo). Normal human epidermal keratinocytes were maintained in keratinocyte complete 154S culture medium supplemented with 5 µg/mL insulin, 0.5 µM hydrocortisone, 5 µg/mL transferrin, 200 pg/mL human recombinant epithelial growth factor, and 0.2% bovine pituitary extract (all purchased from Kurabo).

Assay of inhibition of melanocyte dendrite outgrowth

Normal human epidermal melanocytes were plated on a 48-well microtiter plate at a density of 3,000 cells per well and treated 24 h later with a plant extract for a total of 48 h. Melanocyte morphology was observed microscopically 24 h and 48 h after treatment. When melanocyte dendrite shrinkage was observed, we confirmed that the shrinkage was not due to cell toxicity by removing the plant extract from the culture medium and observing the melanocytes.

Assay of expansion of melanocyte dendrites

Normal human epidermal melanocytes were maintained in a culture medium lacking phorbol-12-myristate-13-acetate and bovine pituitary extract and then plated on a 48-well microtiter plate at a density of 3,000 cells per well. After 24 h incubation, plant extracts were added to each well. The microtiter plate was incubated for 48 h and melanocyte morphology was observed.

Isolation of methylophiopogonanone B from ophiopogon tuber

Methylophiopogonanone B (5,7-dihydroxy-6,8-dimethyl-3-(4-methoxybenzyl)-chroman-4-one) and methylophiopogonanone A (5,7-dihydroxy-6,8-dimethyl-3-(3,4-methylenedioxybenzyl)-chroman-4-one) were isolated from *Ophiopogon japonicus* as follows. Methanol extracts of ophiopogon tuber were evaporated to dryness *in vacuo*. The residue was dissolved in water, extracted with ethyl acetate and concentrated under reduced pressure. The residue was eluted through a column of silica gel with chloroform to give a mixture of methylophiopogonanone A and B. The mixture was purified by Sephadex LH-20 column chromatography using methanol as the eluent. Methylophiopogonanone B was identified by ^{1}H-NMR and ^{13}C-NMR, the results of which were in agreement with previously published data [9].

Isolation of centaureidin from Achillea millefolium

Centaureidin (5,7,3'-trihydroxy-3,6,4'-trimethoxyflavone) was isolated from *Achillea millefolium*. The leaves were extracted with methanol and concentrated *in vacuo*. The residue was poured into water and extracted with ethyl acetate. The organic layer was concentrated under reduced pressure, and the residue was purified by silica gel chromatography using chloroform as the eluent to give pure centaureidin that was subsequently crystallized from chloroform. Analyses by ^{1}H-NMR, ^{13}C-NMR, EI-MS, and X-ray crystallography proved that our extract was in fact centaureidin, as our results were in agreement with previously published data [10].

Isolation of methylswertianin from the swertia herb

Methylswertianin (1,8-dihydroxy-3,7-dimethoxyxanthone) was isolated from the swertia herb. The leaves were extracted with methanol and the solvent evaporated under reduced pressure. The extract was suspended in distilled water and extracted with ethyl acetate. The ethyl acetate layer was concentrated under reduced pressure and then subjected to silica gel column chromatography and elution with chloroform to yield a crude fraction that included methylsweratianin. This fraction was repeatedly purified by silica gel chromatography using chloroform as the eluent to obtain pure methylsweratianin. Methylsweratianin was identified by ^{1}H-NMR and ^{13}C-NMR, the results of which were in agreement with previously published data [11].

Preparation of comfrey extract

Comfrey was extracted with methanol and concentrated under reduced pressure to produce a dark green viscous mass which was subjected to silica gel column chromatography. The column was developed with 0%, 5%, 10%, 20%, and 30% methanol in chloroform to obtain the various fractions. All these fractions were tested for their ability to expand melanocyte dendrites; only the 30% methanol in chloroform eluted fraction showed potent activity.

Cytotoxicity of methylophiopogonanone B and centaureidin in normal human epidermal melanocytes

Cytotoxicity was assessed using a commercially available kit (Cell Counting Kit-8; Dojindo Co. Kumamoto, Japan) containing the highly water soluble tetrazolium salt, WST-8, (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Normal human epidermal melanocytes were plated on 96-well microtiter plates at a density of 10,000 cells per cm^2. After 24 or 48 h in the presence of various concentrations of methylophiopogonanone B or centaureidin, cells were assayed according to the manufacture's protocol and incubated for 2 h at 37 °C. Absorbance was measured at 450 nm using a microplate reader (Benchmark Plus; BioRad, Hercules, CA).

Measurement of melanin production

This part of the experiment was done according to the method described by Whittaker [12]. Briefly, normal human epidermal melanocytes were incubated with 0.0185 MBq [^{14}C] thiouracil and 1 µM methylophiopogonanone B or 0.5 µM centaureidin for 5 days, then trypsinized and precipitated by centrifugation. Next, the pelleted cells were precipitated with 10% trichloroacetic acid and then lysed in scintillation cocktail. Radioactivity was measured using a liquid scintillation counter (LSC6100; ALOKA, Tokyo, Japan). The calculated amount of radioactivity was equilibrated using the viable cells ratio from the WST-8 assay.

Scanning laser confocal microscopy

Cells stained with the succinimidyl ester of carboxy fluorescein diacetate (CFDA) (Molecular Probes, Eugene, Oregon) were scanned according to the procedure described by Minwalla *et al.* [13]. Briefly, normal human epidermal melanocytes stained with 2 µM CFDA were co-cultured with normal human epidermal keratinocytes in a ratio of 1:2. For various periods of time the co-cultures were maintained in complete melanocyte medium and complete keratinocyte culture medium in a ratio of 1:2. The cells were then fixed with 4% formalin in phosphate-buffered saline and mounted with fluoromount G (Southern Biotechnology Associates Inc., Birmingham, Alabama). The CFDA transferred from the melanocytes to the keratinocytes was observed using an Olympus BX51 laser microscope (Olympus, Tokyo, Japan).

Histochemistry of reconstructed epidermis

The reconstructed human epidermis (Kurabo) consisted of normal human-derived epidermal keratinocytes and melanocytes that had been cultured to form a multilayered, highly differentiated model. Reconstructed epidermis was incubated in medium containing the specific agents being tested. The medium and agents were replaced every other day. After treatment the epidermis sheets were prepared, and skin sections were processed for histochemical Fontana Masson staining.

RESULTS AND DISCUSSION

Shrinkage of normal human melanocyte dendrites

Effects of methylophiopogonanone B and centaureidin on normal human epidermal melanocytes morphology

From among the large number of plant extracts that we screened, ophiopogon tuber and *Achillea millefolium* extracts were found to be most effective in shrinking normal human melanocyte dendrites. Further investigation revealed that methylophiopogonanone B, a component of the ophiopogon tuber extract, and centaureidin, a component of the *Achillea millefolium* extract ***(Figure 1)***, were responsible for the observed effects ***(Figure 2)***. We ascertained that the shrinkage was not due to cell toxicity because removal of methylophiopogonanone B and centaureidin from the culture medium after 48 hours of treatment resulted in the elongation of the melanocyte dendrites to a normal state without any apparent damage 24 h later ***(Figure 2H and L)***.

Cytotoxicity of methylophiopogonanone B and centaureidin in normal human epidermal melanocytes

We examined the viability of normal human epidermal melanocytes monocultures after exposure to various concentrations of methylophiopogonanone B and centaureidin. Methylophiopogonanone B and centaureidin shrank dendrites at concentrations of 1 µM and 0.5 µM, respectively. Methylophiopogonanone B (1 µM)

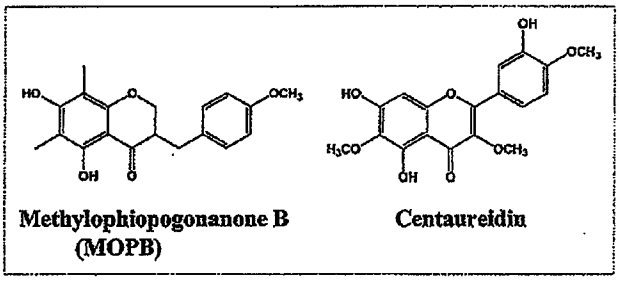

Figure 1: *Structures of methylophiopogonanone B (MOPB) and centaureidin, both of which cause shrinkage of normal human melanocyte dendrites*

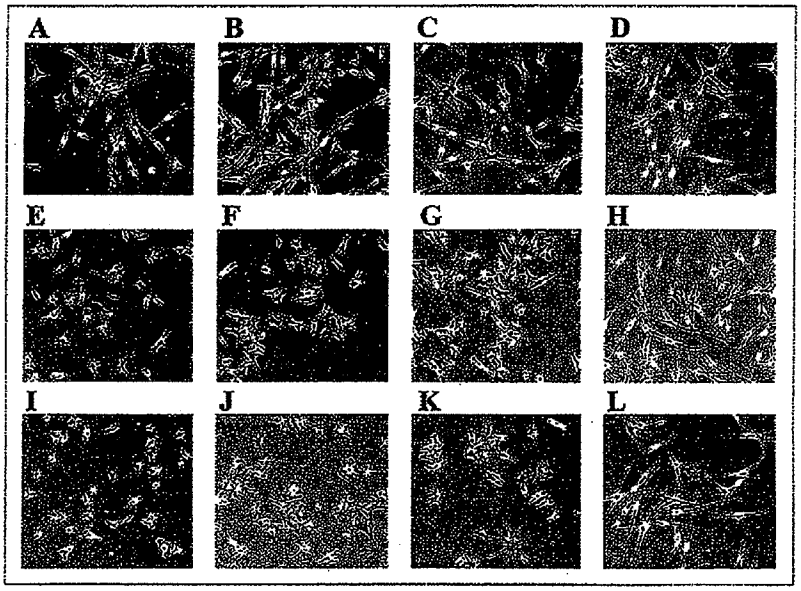

Figure 2: *The effects of methylophiopogonanone B (MOPB) and centaureidin on melanocyte morphology. A, B, C, and D treated with DMSO blank, (A) 24 h treatment, (B) 48 h treatment, (C) 72 h treatment, (D) 24 h after removing DMSO from the medium after 48 h treatment. E, F, G, and H treated with 1 µM methylophiopogonanone B in DMSO, (E) 24 h treatment, (F) 48 h treatment, (G) 72 h treatment, (H) 24 h after removing methylophiopogonanone B from the medium after 48 h treatment. I, J, K, and L treated with 0.5 µM centaureidin in DMSO, (I) 24 h treatment, (J) 48 h treatment, (K) 72 h treatment, (L) 24 h after removing centaureidin from the medium after 48 hours of treatment.*

and centaureidin (0.5 μM) showed no toxicity to the normal human melanocytes based on the WST-8 assay method ***(Figure 3)***. Thus, the dendrite retraction induced by methylophiopogonanone B and centaureidin appeared to be reversible and not associated with toxicity. Methylophiopogonanone B and centaureidin would be safe and effective for shrinkage of normal human epidermal melanocytes.

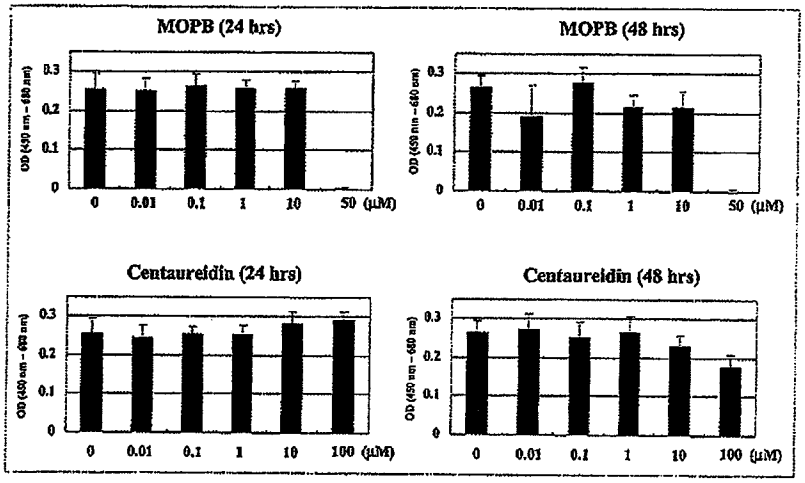

Figure 3: *Cytotoxicity of methylophiopogonanone B (MOPB) and centaureidin in normal human epidermal melanocytes. Cell viability was examined at 24 and 48 h after culture at the indicated concentrations of methylophiopogonanone B and centaureidin. Each value represents the mean OD 450 nm minus OD 680 nm of 6 determinations ± standard deviation.*

Effects of methylophiopogonanone B and centaureidin on melanin synthesis

The dynamic changes in melanocyte cell morphology should have an influence on the basic function of melanocytes, including melanogenesis. Melanin synthesis was assessed in normal human epidermal melanocytes after exposure to methylophiopogonanone B (1 μM) or centaureidin (0.5 μM) for 5 days. Melanin synthesis was reduced slightly by dendrite shrinkage with the addition of methylophiopogonanone B and centaureidin ***(Figure 4)***.

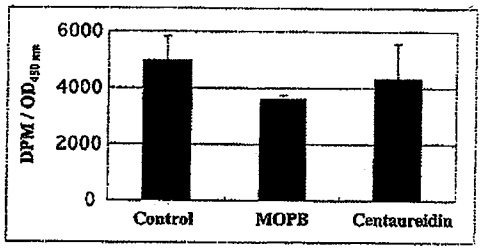

Figure 4: *The effects of methylophiopogonanone B (MOPB) and centaureidin on melanin synthesis. Melanin production and cell viability were assessed after 5 days in the presence of methylophiopogonanone B (1 μM) or centaureidin (0.5 μM). Each value represents the mean amount of radioactivity per viable cell of 3 determinations ± standard deviation.*

Effects of methylophiopogonanone B and centaureidin on inhibition of melanosome transfer due to dendrite shrinkage

Melanocytes transfer melanosomes through their dendrites to surrounding keratinocytes in the skin. To study the effects of methylophiopogonanone B and centaureidin on melanosome transfer, co-cultures of normal human melanocytes and keratinocytes were used to test methylophiopogonanone B and centaureidin for their ability to reduce melanosome transfer by shrinking the dendrites. The transfer of fluorochrome from labeled melanocytes to keratinocytes in an *in vitro* model system has been observed [13]. CFDA-labeled melanocytes were co-cultured with keratinocytes in the presence of methylophiopogonanone B (1 μM) or centaureidin (0.5 μM) for 5 days. The DMSO-treated cells were almost all keratinocytes that contained fluorochrome obtained from surrounding melanocytes ***(Figure 5D)***. Methylophiopogonanone B and centaureidin effectively inhibited the transfer of fluorochrome to the keratinocytes by causing dendrite shrinkage ***(Figure 5E and 5F)***. Our results demonstrated that melanosome transfer can be interrupted *in vitro* by adding methylophiopogonanone B or centaureidin to co-cultures of melanocytes and keratinocytes. Hakozaki *et al.* reported that niacinamide (vitamin B_3) suppresses melanosome transfer, and in their human clinical study topical application of niacinamide produced a remarkable reduction of cutaneous pigmentation, including moderate lentigo senilis melasma and freckles [14], suggesting that a drug which inhibits melanosome transfer could be a breakthrough agent for reducing melanin in the epidermis. Cells treated with niacinamide (1 mM) transferred a small amount of fluorochrome to keratinocytes (data not shown). The inhibition of melano-some transfer by niacinamide was reported to be approximately 35-68% [14]. On the other hand, melanocytes treated with methylophiopogonanone B or centaureidin did not transfer fluorochrome to keratinocytes, and all of the dye remained in the melanocytes ***(Figure 5E and F)***. Thus, methylophiopogonanone B and centaureidin appeared to block melanosome transfer completely, whereas niacinamide did so only partially.

Effects of methylophiopogonanone B and centaureidin on melanocytes maintained in a three-dimensional culture model

Further studies were performed on the effects of methylophiopogonanone B and centaureidin on melanocytes maintained in a three-dimensional culture model. After 12 days of treatment the reconstructed epidermis was observed for melanocyte morphology and melanin distribution. To study melanocyte morphology, the prepared epidermis sheets were processed for Fontana Masson staining. Based on ultrastructure studies, methylophiopogo-

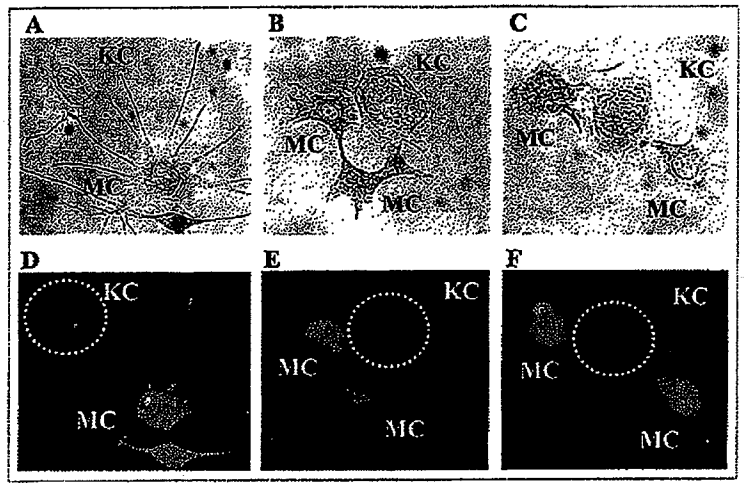

Figure 5: The effects of methylophiopogonanone B (MOPB) and centaureidin on inhibition of melanosome transfer due to dendrite shrinkage – confocal images of co-cultures of CFDA-labeled melanocytes (MC) with keratinocytes (KC). Differential interference contrast images are shown above each confocal image to depict the interaction between the melano-cytes and keratinocytes. A and D treated with DMSO blank for 5 days, B and E treated with 1 µM methylophiopogonanone B in DMSO for 5 days, C and F treated with 0.5 µM centaureidin in DMSO for 5 days.

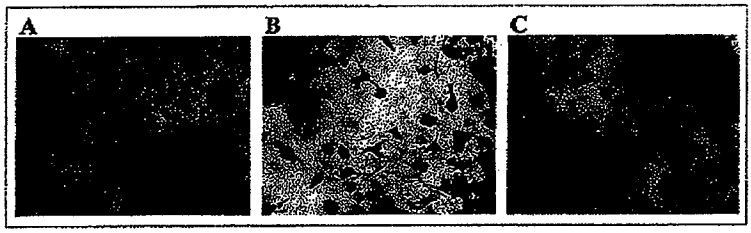

Figure 6: The effects of methylophiopogonanone B (MOPB) and centaureidin on melanocytes maintained in a three-dimensional culture model. After 12 days of treatment the morphology of melanocytes was observed. Epidermis sheets were processed for Fontana Masson staining. (A) treated with DMSO blank, (B) treated with 2 µM methylophiopogonanone B in DMSO, (C) treated with 3 µM centaureidin in DMSO.

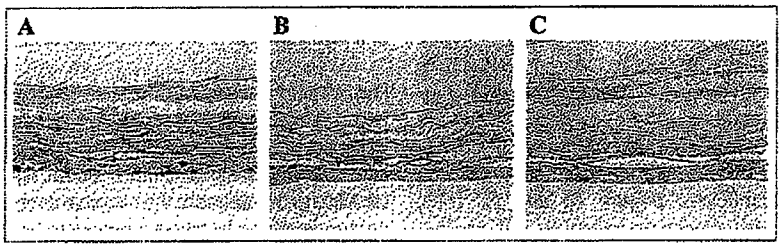

Figure 7: Effects of methylophiopogonanone B (MOPB) and centaureidin on melanocytes maintained in a three-dimensional culture model. After 12 days of treatment cultured skin sections were processed for histochemical Fontana Masson staining. (A) treated with DMSO blank, (B) treated with 2 µM methylophiopogonanone B in DMSO, (C) treated with 3 µM centaureidin in DMSO.

nanone B and centaureidin were found to induce shrinkage of melanocyte dendrites ***(Figure 6)***. To study melanin distribution, cultured skin sections were processed for histochemical Fontana Masson staining. Methylophiopogonanone B and centaureidin were shown to clearly inhibit melanosome transfer to keratinocytes through dendrite shrinkage in the three-dimensional culture model ***(Figure 7)***.

Expansion of normal human melanocyte dendrites

Identification of methylswertianin

Encouraged by the discovery of the plant extracts which could effectively shrink melanocyte dendrites, we decided to search for extracts which would have the opposite effect, namely expanding dendrites to produce more melanin. After screening a large number of extracts for this purpose, we discovered that swertia herb and comfrey extracts are the most effective. Further evaluation revealed that methylswertianin ***(Figure 8)***, a component of swertia herb extract, was responsible for the observed effects of the swertia herb extract.

Effects of methylswertianin and comfrey extract on the promotion of melanosome transfer due to dendrite expansion

To study the effects of methylswertianin and comfrey extract on melanosome transfer, co-cultures of normal human melanocytes and keratinocytes were used to test methylswertianin and comfrey extract for the promotion of melanosome transfer due to dendrite expansion. CFDA-labeled melanocytes were co-cultured with keratinocytes in the presence of methylswertianin or comfrey extract. Methylswertianin and comfrey extract effectively increased the transfer of the fluorochrome to keratinocytes due to dendrite expansion ***(Figure 9)***. Our results demonstrated that melanosome transfer can be promoted *in vitro* by adding methylswertianin or comfrey extract to co-cultures of melanocytes and keratinocytes. Seiberg *et al.* reported that the protease-activated receptor 2 (PAR-2) expressed on keratinocytes, but not on melanocytes, is involved in melanosome transfer and

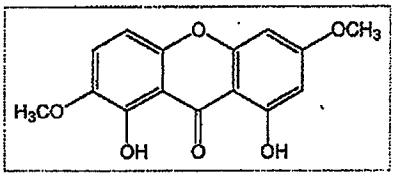

Figure 8: Structure of methylswertianin, which causes expansion of normal human epidermal melanocytes dendrites.

therefore may regulate pigmentation. They suggested the regulation of pigmentation, mediated by the activation or inhibition of the keratinocyte receptor PAR-2 [15]. As our work focused on the melanocyte dendrites, we did not demonstrate that the PAR-2 expressed or suppressed on keratinocytes by adding methylophiopogonanone B, centaureidin, methylswertianin or comfrey extract. We also did not investigate whether these agents affected other dendritic cells such as Langerhans cell.

Effects of methylswertianin and comfrey extract on melanocytes maintained in a three-dimensional culture model

Further studies were performed on the effects of methylswertianin and comfrey extract on melanocytes maintained in a three-dimensional culture model. After 9 days of treatment, epidermis sheets were processed for Fontana Masson staining. Based on ultrastructure studies, methylswertianin and comfrey extract were found to induce expansion of melanocyte dendrites ***(Figure 10)***.

CONCLUSION

Our experimental findings suggest the possibility of manipulating human skin color by controlling melanosome transfer through the shrinkage or expansion of dendrites. Our work involved the use of human melanocytes and keratinocytes. We found that methylophiopogonanone B and centaureidin, active components present in ophiopogon tuber and *Achillea millefolium* extract, can induce shrinkage of melanocytes, thus causing a reduction in melanosome transfer to keratinocytes, which suppresses skin pigmentation. Furthermore, we found that methylswertianin and comfrey extract have the opposite effect by promoting the expansion of melanocyte dendrites, thereby increasing melanosome transfer, which increases skin pigmentation.

We focused our investigation on botanical extracts since they are known to have a relative low cytotoxicity and to be distributed all over world. Both chemicals and plant extracts including active materials produced the desired effects such as shrinkage or expansion of melanocyte dendrites. A combination of effective agents, in addition to the ones identified in this work, could result in the creation of very unique cosmetic products that precisely control the darkening or lightening of skin tone.

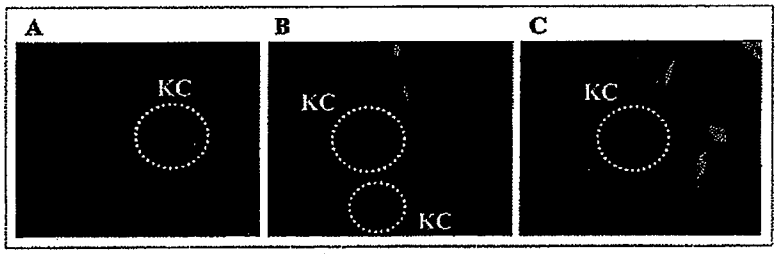

Figure 9: The effects of methylswertianin and comfrey extract on the promotion of melanosome transfer due to dendrite expansion – confocal images of co-cultures of CFDA-labeled melanocytes with keratinocytes (KC). (A) treated with DMSO blank for 6 days, (B) treated with 0.2 mM methylswertianin in DMSO for 6 days, (C) treated with 0.001% comfrey extract in DMSO for 6 days.

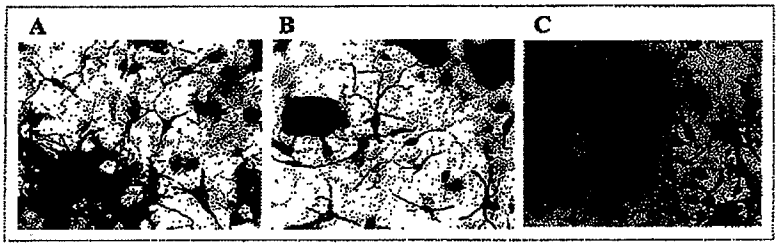

Figure 10: The effects of methylswertianin and comfrey extract on melanocytes maintained in a three-dimensional culture model. After 9 days of treatment melanocyte morphology was examined. Epidermis sheets were processed for Fontana Masson staining. (A) treated with DMSO blank, (B) treated with 0.4 mM methylswertianin in DMSO, (C) treated with 0.001% comfrey extract in DMSO.

Acknowledgements

We would like to thank Ms. Kazuko Nishikawa for her assistance with the histological studies.

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EXHIBIT E

TOYA107.007APC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: TADA et al.	
	Art Unit: 1612
Application No.: 10/537,320	Examiner: SZNAIDMAN, MARCOS L
Filing Date: June 2, 2005	Attorney DOCKET No.: TOYA107.007APC
For: DENDRITE ELOGATION INHIBITOR FOR MELANOCYTE AND SKIN PREPARATION FOR EXTERNAL USE CONTAINING THE SAME	

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O.Box 1450
Alexandria, VA 22313-1450
Dear Sir:

I declare as follows:

1. I am employee of Pola Chemical Industries Inc. located at 6-48, Yayoi-cho, Suruga-ku, Shizuoka-shi, Shizuoka, Japan, which is engaged in the business of production and sale of cosmetics.

2. I am one of co-inventors of the above-identified patent application.

3. Ishida et al. (EP 1147764) describes that a compound represented by the general formula I is used for the treatment of skin problems like skin darkening, wrinkles, etc. However, the compound described in Ishida et al. does not inhibit elongation of melanocytic dendrite as opposed to Centaureidin of the amended Claim 7 in the present application.

4. I have conducted the experiments described herein and present them as evidence supporting the above fact.

TOYA107.007APC

5. Purpose of the experiments

Comparing an inhibitory action on the elongation of melanocytic dendrites between Centaureidin and a compound represented by the formula III (Nobiletin) disclosed in Ishida et al.

6. Method of the experiments

Inhibitory actions on the elongation of melanocyic dendrites were examined for Centaureidin and Nobiletin according to the method described in Example 1 of the present description. Note that a control was a solution having only a basal medium.

The concentration of the compound in each Sample was as follows.

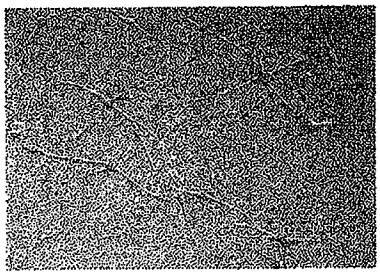
Sample 1 Centaureidin 10^{-5}%
Sample 2 Nobiletin 10^{-4}%
Sample 3 Nobiletin 10^{-5}%
Sample 4 Nobiletin 10^{-6}%

7. Results

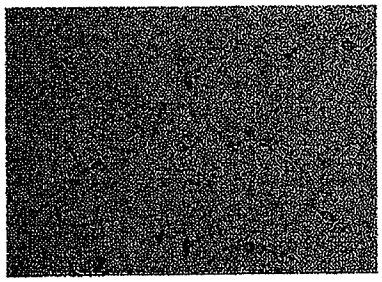
The results are shown in the following table and photographs. The table shows length of the dendrite.

	Added compound	Length of dendrite (μm)
Sample 1	Centaureidin 10^{-5}%	30±10
Sample 2	Nobiletin 10^{-4}%	139±23
Sample 3	Nobiletin 10^{-5}%	142±18
Sample 4	Nobiletin 10^{-6}%	141±30
Control	-	143±35

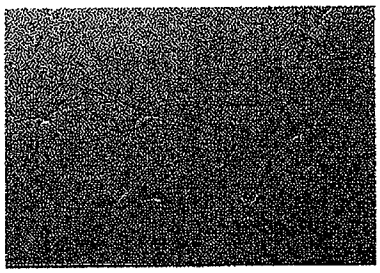
TOYA107.007APC



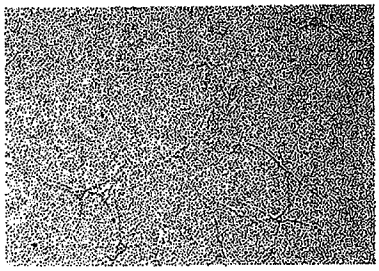
Control



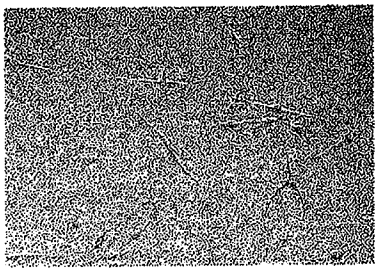
Sample 1
(Centaureidin 10^{-5}%)



Sample 2
(Nobiletin 10^{-4}%)



Sample 3
(Nobiletin 10^{-5}%)



Sample 4
(Nobiletin 10^{-6}%)

It is clear that the dendrites were elongated in the added groups of Nobiletin as well as the control, while the dendrites were not elongated in the added group of Centaureidin.

8. I further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Akihiro Tada
Akihiro TADA

Date: April 13, 2009

EXHIBIT D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: TADA et al.	
	Art Unit: 1612
Application No.: 10/537,320	Examiner: SZNAIDMAN, MARCOS L
Filing Date: June 2, 2005	Attorney DOCKET No.: TOYA107.007APC
For: DENDRITE ELONGATION INHIBITOR FOR MELANOCYTE AND SKIN PREPARATION FOR EXTERNAL USE CONTAINING THE SAME	

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O.Box 1450
Alexandria, VA 22313-1450
Dear Sir:

I declare as follows:

1. I am employee of Pola Chemical Industries Inc. located at 6-48, Yayoi-cho, Suruga-ku, Shizuoka-shi, Shizuoka, Japan, which is engaged in the business of production and sale of cosmetics.

2. I am one of co-inventors of the above-identified patent application.

3. The actions of Centaureidin and Nobiletin (EP 1147764) to melanocyte are clearly different from each other. Nobiletin inhibits production of melanin, however, Centaureidin does not inhibit production of melanin.

4. I have conducted the experiments described herein and present them as evidence supporting the above fact.

5. Test Example: Test on Inhibition of Melanin Production using Cultured Normal Human Melanocyte

Inhibitory actions of Centaureidin and Nobiletin on melanin production

was evaluated by using 2-thiouracil (^{14}C-labelled 2-thiouracil in this test) specifically incorporated into melanin in the process of intracellular synthesis of melanin.

A 24-well microplate was used and 2 ml of complete medium for culturing melanocytes (product of KURABO INDUSTRIES LTD.) was added to each of 21 wells of the plate. Into each of the 21 wells, normal human melanocytes (product of KURABO INDUSTRIES LTD.) were inoculated at a concentration of 1.5×10^4 cells/cm^2 and cultured at 37°C in 5% carbon dioxide atmosphere for 24 hours.

Subsequently, the media of all of the above 21 wells were replaced as follows:

in respect of three wells, the medium was replaced by new complete medium for culturing melanocytes (control wells);

in respect of nine wells, the medium was replaced by complete medium for culturing melanocytes containing Centaureidin at concentrations of 0.1 μM, 0.5μM and 1 μM each for three wells; and

in respect of another nine wells, the medium was replaced by complete medium for culturing melanocytes containing Nobiletin at concentrations of 1 μM, 5 μM and 10 μM each for three wells.

Further, 2-[2-^{14}C]thiouracil (^{14}C-labelled thiouracil) was added at 0.25×10^{-6}Ci (curie). The cells were further cultured under the same conditions as above for three days. After the cultivation was completed, the culture broth was removed from each of the wells, and each of the wells was washed with PBS (phosphate buffered saline). Thereafter, cells were peeled off from the bottom surfaces of wells by using a medium containing trypsin and EDTA to thereby prepare a cell-suspension liquid, and cells were collected from the liquid by centrifugation. The number of the cells was counted by using a hemocytometer. Subsequently, the amount of 2-[2-^{14}C]thiouracil (0.25×10^{-6}Ci) contained in the cells collected from each of the wells was measured by using a liquid scintillation counter (product of ALOKA CO., LTD.). The percentage of the radiation dose detected from the cells cultured in each of the media containing a substance to be evaluated against the radiation dose detected from the cells collected from the control wells was calculated and defined as melanin amount(%). That is, it can be assumed that the smaller the radiation dose incorporated in each cell is, the smaller the melanin amount is, and thus the higher the inhibitory action of the added substance is.

6. Results

The results are shown in the following table.

Nobiletin inhibits production of melanin, however, Centaureidin does not inhibit production of melanin.

Compound(concentration)	Melanin amount(%)
Centaureidin (0.1 μM)	101.36 ± 3.57
Centaureidin (0.5 μM)	102.68 ± 6.52
Centaureidin (1 μM)	100.86 ± 5.41
Nobiletin (1 μM)	83.67 ± 5.77
Nobiletin (5 μM)	70.03 ± 5.06
Nobiletin (10 μM)	53.62 ± 6.35

7. I further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Akihiro Tada.

Akihiro TADA

Date: June 24 , 2010

DYSCHROMATOSIS ATTACHMENT

EXHIBIT E

online medical dictionary from mondofacto

dyschromatosis medical dictionar

An asymptomatic anomaly of pigmentation occurring among the Japanese; may be localised or diffuse.

Origin: dys-+ G. Chroma, colour, + -osis, condition

(05 Mar 2000)

dyschondroplasia with haemangiomas, dyschondrosteosis, dyschroia, dyschromatopsia < Prev | Next > dyschromia, dyscinesia, dyscoimesis

EXHIBIT F

OP1650

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European Patent Office
Office européen des brevets

(11) EP 1 147 764 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
24.10.2001 Bulletin 2001/43

(51) Int Cl.[7]: **A61K 7/48**, C07D 311/30

(21) Application number: **01109611.2**

(22) Date of filing: **18.04.2001**

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
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(30) Priority: **19.04.2000 JP 2000117519**

(71) Applicant: **NOF CORPORATION**
Tokyo (JP)

(72) Inventors:
- **Ishida, Misaki**
  Amagasaki-shi, Hyogo (JP)
- **Sato, Saori**
  Minamiashigara-shi, Kanagawa (JP)
- **Hashizume, Ron**
  Abiko-shi, Chiba (JP)
- **Hayashi, Shinji**
  Amagasaki-shi, Hyogo (JP)

(74) Representative: **Koepe, Gerd L., Dipl.-Chem.**
Koepe, Flesser & Partner,
Radeckestrasse 43 EG
81245 München (DE)

(54) Cosmetic composition

(57) A cosmetic composition comprises 0.00005 to 10wt% of polymethoxyflavone having at least four methoxy groups. Cosmetic compositions in the form of lotions, milky lotions, oil-in-water creams, water-in-oil creams are preferable. Thus, the present invention provides cosmetic compositions that are excellent in the whitening effect, allow the skin to retain moisture for a long time, vitalize the skin, suppress wrinkles, and have excellent storage stability.

EP 1 147 764 A2

Printed by Jouve, 75001 PARIS (FR)

Description

BACKGROUND OF THE INVENTION

1. Field of the invention

[0001] The present invention relates to cosmetic compositions. More specifically, the present invention relates to cosmetic compositions that are excellent in whitening effects, effectively prevent "liver spots", "freckles" etc. that are caused by sunburn, allow the skin to retain moisture for a long time, vitalize the skin, suppress wrinkles, and are excellent in safety and storage stability.

2. Description of the prior art

[0002] When the skin is irradiated with ultraviolet rays (hereinafter referred to as UV), melanocytes in the skin are activated, so that synthesis of melanin is promoted by the enzyme tyrosinase as well as TRP1 and TRP2. Deposition of the produced melanin in the skin results in "liver spots" or "freckles", and therefore various cosmetic compositions, especially whitening cosmetics are used to prevent such melanin production. Moreover, the UV are found to promote oxidation of sebum cutaneum, cell membranes or the like and cause various skin disorders. In recent years, in particular, an increase in the amount of UV due to damage of the ozone layer is noted as a problem, and it is desired to prevent oxidation in the skin more effectively.

[0003] Ascorbic acid phosphate ester salts, hydroquinon derivatives, placental extracts, kojic acids, ellagic acid or the like are known as components exhibiting a whitening effect, and cosmetic compositions comprising these components are commonly used. Among these, at present, the components that are most commonly used are placental extracts, usually bovine placental extracts. However, in recent years, bovine spongiform encephalopathy has become worldwide problems. In Japan, use of bovine placental extracts is banned since December 2000. Therefore, a novel and effective whitening component is in demand.

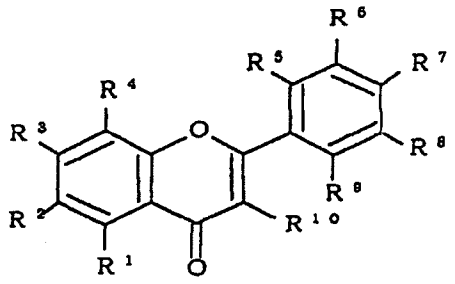
[0004] On the other hand, in recent years, it has been reported that polyphenol or the like contained in plants has a high whitening effect, and cosmetic compositions employing it have been proposed. Furthermore, for example, Japanese Laid-Open Patent Publication (Tokkai) No. 6-16531 reports that flavanones have a whitening effect, and Japanese Laid-Open Patent Publication (Tokkai) No. 10-101543 reports that hydroxyflavones have a whitening effect. However, not only are the effects of these whitening components not sufficient yet, but also these components have poor storage stability, so that when they become finished products, various problems may arise over time. Furthermore, although these whitening components have a whitening effect, so-called anti skin-aging effects such as vitalizing the skin and preventing wrinkles cannot sufficiently be provided at present.

[0005] Therefore, there is a great demand for cosmetic compositions having an excellent whitening effect, providing a skin aging prevention effect, high safety in use, and excellent storage stability.

SUMMARY OF THE INVENTION

[0006] Therefore, it is an object of the present invention to provide a cosmetic composition having a sufficient excellent whitening effect, a so-called anti skin-aging effect such as vitalizing the skin and preventing wrinkles, high safety in use, and excellent storage stability. This object can be achieved by blending a specific amount of a specific polymethoxyflavone compound.

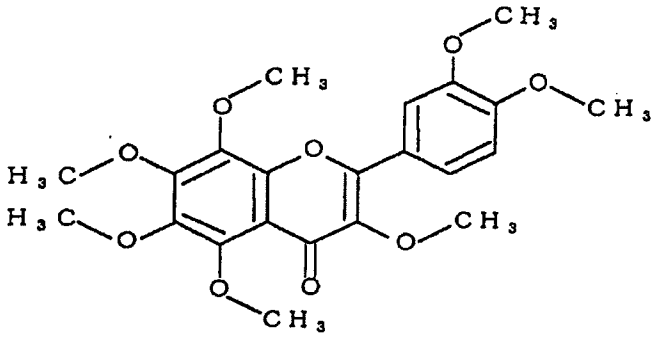
[0007] A cosmetic composition of the present invention includes 0.00005 to 10 percent by weight (hereinafter referred to as wt%) of polymethoxyflavone represented by formula (I):



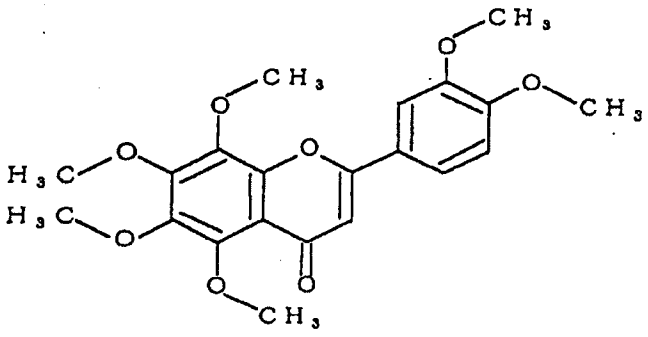
(I)

wherein each of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} is selected from the group consisting of hydrogen atom, hydroxyl group, alkoxy group having 1 to 20 carbon atoms, alkyl group having 1 to 20 carbon atoms, alkenyl group having 2 to 20 carbon atoms, hydroxyalkyl group having 1 to 20 carbon atoms or a sugar residue, and at least four of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are methoxy groups.

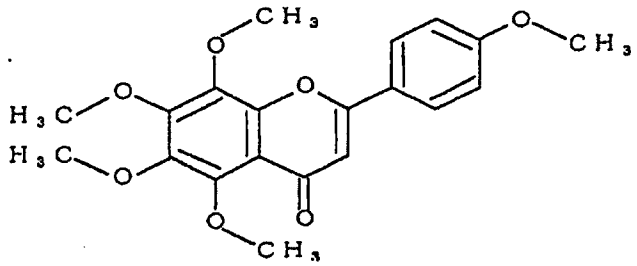
[0008] In one preferred embodiment of the present invention, the polymethoxyflavone comprises at least one compound selected from the group consisting of compounds represented by formulae (II) to (V):



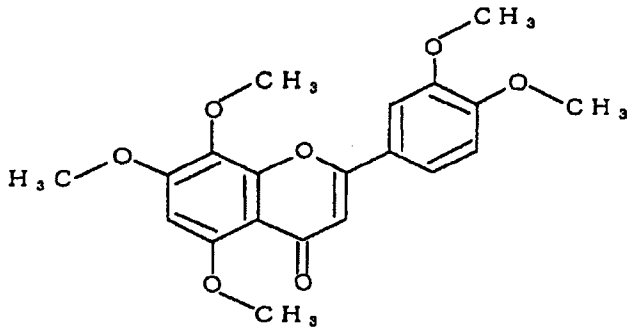
(II)



(III)



(IV)



(V)

[0009] In one preferred embodiment of the present invention, the polymethoxyflavone comprises at least one compound selected from the group consisting of 5,6,7,8,3',4'-hexamethoxyflavone and 5,6,7,8,4'-pentamethoxyflavone.

[0010] According to another aspect of the present invention, a method for isolating and purifying polymethoxyflavone includes the steps of: subjecting peel of a plant of the Genus Citrus of the Family Rutaceae to extraction with at least one solvent selected from the group consisting of methanol, ethanol, propanol, butanol, ethyl acetate, acetone, propylene glycol, and 1, 3-butylene glycol to obtain an extract (S1); dissolving the extract (S1) in ethyl acetate, adding water thereto, stirring, separating into layers, removing a water layer, distilling off the ethyl acetate to obtain a dry solid product (S2); and dissolving the dry solid product (S2) in a solvent, and subjecting it to liquid column chromatography.

[0011] According to another aspect of the present invention, a method for isolating and purifying polymethoxyflavone comprising the steps of: subjecting peel of a plant of the Genus Citrus of the Family Rutaceae to extraction with at least one solvent selected from the group consisting of methanol, ethanol, propanol, butanol, ethyl acetate, acetone, propylene glycol, and 1, 3-butylene glycol to obtain an extract (S1); dissolving the extract (S1) in hexane and/or chloroform, removing a precipitate, distilling hexane and/or chloroform to obtain a dry solid product (S3); and dissolving the dry solid product (S3) in a solvent, and subjecting it to liquid column chromatography.

[0012] In one preferred embodiment of the present invention, the liquid column chromatography uses silica gel and/or alumina as a filler, and a mixed solution of hexane/ethanol in a volume proportion of 70/30 to 97/3 as an eluent.

[0013] Thus, the cosmetic compositions of the present invention has an excellent whitening effect and has excellent storage stability.

[0014] These and other advantages of the present invention will become apparent to those skilled in the art upon reading and understanding the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

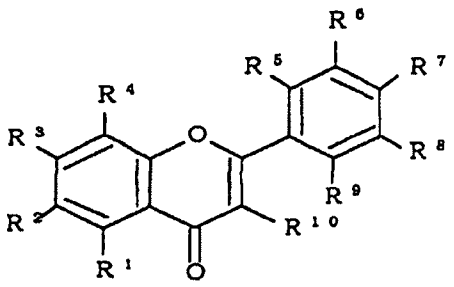
[0015]

Fig. 1 is a diagram showing the ^{1}H-NMR of the compound represented by formula (III).
Fig. 2 is a diagram showing the ^{13}C-NMR of the compound represented by formula (III).
Fig. 3 is a diagram showing the mass spectrum of the compound represented by formula (III).
Fig. 4 is a diagram showing the ^{1}H-NMR of the compound represented by formula (IV).

Fig. 5 is a diagram showing the ^{13}C-NMR of the compound represented by formula (IV).
Fig. 6 is a diagram showing the mass spectrum of the compound represented by formula (IV).

DETAILED DESCRIPTION OF THE INVENTION

[0016] A polymethoxyflavone used in the present invention is represented by the following general formula (I).



(I)

wherein each of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} is selected from the group consisting of hydrogen atom, hydroxyl group, alkoxy group having 1 to 20 carbon atoms, alkyl group having 1 to 20 carbon atoms, alkenyl group having 2 to 20 carbon atoms, hydroxyalkyl group having 1 to 20 carbon atoms or a sugar residue, and at least four of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are methoxy groups. The structural feature is that the second and third positions of the flavonoid skeleton are reduced, and the compound has a total of four or more methoxy groups in the chromone ring and the benzene ring bonded to the second position. In other words, at least four of R^1 to R^{10} are methoxy groups. The positions of the methoxy groups may be either in the chromone ring or the benzene ring, but it is preferable that a larger number of methoxy groups are present in the chromone ring.

[0017] Examples of groups other than the methoxy groups include the following. As the alkoxy group having 1 to 20 carbon atoms, lower alkoxy groups having 1 to 6 carbon atoms are preferable, and methoxy groups, ethoxy groups, propoxy groups or the like are preferable.

[0018] As the alkyl group having 1 to 20 carbon atoms, lower alkyl groups having 1 to 6 carbon atoms are preferable, and methyl groups, ethyl groups, propyl groups or the like are preferable.

[0019] As the alkenyl group having 2 to 20 carbon atoms, lower alkenyl groups having 2 to 6 carbon atoms are preferable, and ethenyl groups, propenyl groups or the like are preferable.

[0020] As the hydroxyalkyl group having 1 to 20 carbon atoms, lower hydroxyalkyl groups having 1 to 6 carbon atoms are preferable, and hydroxymethyl groups, hydroxyethyl groups, hydroxypropyl groups or the like are preferable.

[0021] As the sugar residue, for example, monosaccharides such as glucose, galactose, fucose, xylose, mannose, rhamnose, fructose, arabinose, lyxose, ribose, allose, altrose, idose, talose, deoxyribose, quinovose and abequose; oligosaccharide residues where 2 to 4 of these monosaccharides are bonded, such as maltose, lactose, cellobiose, raffinose, xylobiose and sucrose can be used. Among these, residues of glucose, galactose, fucose, xylose, mannose, rhamnose, fructose are preferable.

[0022] Specific examples of the polymethoxyflavone include 3,5,6,7,8,3',4'-heptamethoxyflavone represented by formula (II), 5,6,7,8,3',4'-hexamethoxyflavone (commonly called nobiletin) represented by formula (III), 5,6,7,8,4'-pentamethoxyflavone (commonly called tangeretin) represented by formula (IV), and 5,7,8,3',4'-pentamethoxyflavone represented by formula (V).

[0023] Among these, 5,6,7,8,3',4'-hexamethoxyflavone (nobiletin) represented by formula (III), and 5,6,7,8,4'-pentamethoxyflavone (tangeretin) represented by formula (IV) are particularly preferable.

[0024] The polymethoxyflavone compound used in the present invention can be obtained by chemical synthesis, or can be obtained as a natural product from an extract with a solvent derived from a plant.

[0025] Examples of chemical synthesis include the method described in Indian Journal of Heterocyclic Chemistry Vol. 6. Jan-March 1997, pp.221-222. With this method, 5,6,7,8,3',4'- hexamethoxyflavone (formula (III):nobiletin) can be synthesized.

[0026] Examples of a method by which polymethoxyflavone is obtained from an extract of a plant include extracting the polymethoxyflavone from the peel of plants of the Genus Citrus of the Family Rutaceae. Plants of the Genus Citrus of the Family Rutaceae, such as Citrus unshiu, Citrus poonensis, Citrus hassaku, Citrus lemon, Citrus tachibana, Citrus

junos, Citrus sudachi, Citrus grandis, Citrus tangerina (tangerine), Citrus reticulate (mandarin orange), Citrus paradisi or the like contains polymethoxyflavone (Journal of Medicinal Plant Research Vol. 46, 162-166, (1982)). Among these, preferable plants are Citrus unshiu, Citrus tachibana, Citrus junos and Citrus sudachi, and more preferable plants are Citrus unshiu and Citrus tachibana.

[0027] Isolation and purification of polymethoxyflavone is performed by producing an extract from the peel of a plant of the Genus Citrus of the Family Rutaceae with an organic solvent, and separating the extract by column chromatography. This method will be described more specifically below.

[0028] First, peel of a plant of the Genus Citrus is subjected to extraction with a water-soluble alcohol such as methanol, ethanol, propanol, butanol, propylene glycol, 1,3-butylene glycol or the like; a solvent such as acetone, hexane, ethyl acetate, chloroform or the like; or a mixed solvent thereof. Among these, at least one solvent selected from the group consisting of methanol, ethanol, propanol, butanol, propylene glycol, 1,3-butylene glycol and ethyl acetate is preferable. More preferable is at least one solvent selected from the group consisting of methanol, ethanol, propylene glycol, 1,3-butylene glycol and ethyl acetate. When the extract liquid is concentrated, an extract (S1) comprising about 1 to 15 wt% of the desired polymethoxyflavone can be obtained. This extract (S1) can be further dried to a solid.

[0029] This extract (S1) is dissolved in ethyl acetate in an amount of twice or more times, preferably, 3 to 10 times the weight of the extract, water is added thereto, the mixture is stirred and separated into layers, and a water layer is removed. Then, the ethyl acetate is distilled off so as to obtain a dry solid product (S2).

[0030] Alternatively, the extract (S1) is dissolved in hexane and/or chloroform, stirred, and is left undisturbed. Thereafter, a precipitate is removed, and the supernatant was concentrated and a dry solid product (S3) can be obtained. As the solvent, hexane or chloroform can be used alone, however, a mixed solvent of hexane/chloroform is preferably used. The mixed solvent can be used in a volume proportion of 1/9 to 9/1, preferably, 3/7 to 7/3.

[0031] The obtained dry solid product (S2) or (S3) is preferably dissolved a suitable solvent and is subjected to liquid column chromatography so that polymethoxyflavone can be isolated and purified. In the liquid column chromatography, silica gel or alumina can be used as a filler, and a mixed solvent of hexane/ethanol in a volume proportion of 70/30 to 97/3 can be used as an eluent.

[0032] More specifically, isolation and purification of polymethoxyflavone from Citrus tachibana will be described. The dried peel of Citrus tachibana (herbal name: "kippi") is pulverized with a blender and dipped in a suitable solvent (e.g., 95% ethanol in an amount of five times the weight) for an appropriate period of time (e.g., five days) for extraction. The extract liquid is filtered and concentrated under reduced pressure so that a kippi extract (corresponding to S1 described above) can be obtained. A suitable amount of ethyl acetate is added to the obtained kippi extract to dissolve the kippi extract, followed by addition of water in an amount equal to that of the ethyl acetate and stirring, and the mixture was left undisturbed. After separating the mixture into layers, a water layer was removed. This operation (washing with water) was repeated plural times, preferably three times or more, and then ethyl acetate was distilled off to obtain a dry solid (corresponding to S2 described above). Alternatively, a suitable amount of mixed solvent of hexane/chloroform (in a volume proportion of 1/1) is added to the obtained kippi extract, and is stored undisturbed over night at an appropriate temperature (e.g., 4 °C). A precipitate is removed by separating means (e.g., centrifugation) and the supernatant is concentrated to obtain a dry solid (corresponding to S3 described above). This dry solid product (corresponding to S2 or S3 described above) is dissolved in an appropriate amount of a suitable solvent (e.g., hexane/ethanol (in a volume proportion of 85/15)), and subjected to fractional liquid chromatography using a silica gel column or an alumina column with a mixed solvent of hexane/ethanol as the eluent for fractionation. Thus, polymethoxyflavone can be isolated and purified.

[0033] The structure of the isolated and purified polymethoxyflavone can be determined by analysis means that is routinely used by those skilled in the art, such as the nuclear magnetic resonance (NMR) spectrum or the mass analysis spectrum.

[0034] In the present invention, the isolated polymethoxyflavone can be added alone or can be used in combination of two or more polymethoxyflavones, or can be added as a mixed product of chemical synthesis or plant extracts that contains a plurality of natural products.

[0035] The polymethoxyflavone is contained in an amount of 0.00005 to 10wt%, preferably 0.0001 to 7wt%, and more preferably 0.001 to 5wt% with respect to the total amount of the cosmetic compound. An amount of below 0.00005 wt% cannot provide a sufficient whitening effect, nor vitalize the skin or suppress wrinkles. An amount of more than 10wt% not only causes a problem in storage stability, but also is expensive.

[0036] Furthermore, the cosmetic composition of the present invention can comprise ascorbic acid and the derivatives thereof, kojic acid and the derivatives thereof, hydroquinone derivatives such as arbutin, placental extracts, ellagic acid and the derivatives thereof, which are known as whitening components. It is preferable that these whitening components are contained in an amount of 0.01 to 10wt%. The whitening effect can be synergistically enhanced by using these whitening components and polymethoxyflavone together.

[0037] The cosmetic composition of the present invention can contain a base, additives or the like that are usually used in cosmetics, as appropriate, depending on the type of the cosmetics in an amount that does not interfere with

the performance of the cosmetic composition. For example, following substances can be contained in the cosmetic composition of the present invention: lower alcohols such as ethanol and isopropyl alcohol; polyhydric alcohols such as glycerol, 1,3-butylene glycol, propylene glycol, dipropylene glycol and polyethylene glycol; hydrocarbon oils such as liquid paraffin, liquid isoparaffin, squalane, veseline and solid paraffin; natural fats and oils such as beef tallow, lard and fish oil; synthetic triglyceride such as glycerol tri 2-ethylhexanoate; ester oils such as isopropyl myristate, isopropyl palmitate, cetyl palmitate, ethyl oleate, oleyl oleate and octyldodecyl myristate; waxes such as yellow bees wax and carnauba wax; silicone derivatives such as linear or cyclic dimethylpolysiloxane, polyether modified dimethylpolysiloxane, amino-modified dimethylpolysiloxane; oils such as ceramide, cholesterol, protein derivatives, lanoline, lanoline derivatives, lecithin; anionic surfactant such as soap, acylmethyl taurine salts, amide ether sulfuric ester salts; amphoteric surfactants such as amideamino acid salts, and amidopropyl dimethylbetaine; nonionic surfactants such as polyoxyethylene alkyl ethers, polyethylene glycol fatty acid esters, sorbitan fatty acid esters, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene hydrogenated castor oil, polyglycerol fatty acid esters, polyoxyethylene glycerol fatty acid esters, glycerol mono-fatty acid esters, alkyl polyglucoside and alkanolamide; cationic surfactants such as alkyltrimethylammonium chloride; semipolar surfactants such as alkyl dimethylamine oxides; water-soluble polymers such as algic acid, carboxyvinyl polymer, carboxymethylcellulose, hydroxypropylmethyl-cellulose, hydroxyethyl cellulose, xanthan gum and hyaluronic acid; organic or inorganic salts such as pyrrolidone carboxylic acid salts, citric acid salts, malic acid salts and sodium chloride; acids or alkalis that are pH regulators; antiinflammatory agents; germicidal agents; chelating agents; antioxidants; ultraviolet absorbers; natural extracts derived from animals and plants; and pigments and fragrances.

[0038] The cosmetic composition of the present invention can be preferably used in the form of lotions, milky lotions, oil-in-water creams, water-in-oil creams or the like.

Examples

[0039] Next, the present invention will be described by way of examples, but the present invention is not limited by these examples.

(Production Example 1: Isolation of polymethoxyflavone)

[0040] First, 10kg of dried peel of Citrus tachibana (herbal name: "kippi") was pulverized with a blender, and immersed for extraction for 5 days in 95% ethanol (first grade) in an amount of 5 times the weight of the dried peel. An extract liquid was filtered and concentrated under reduced pressure, and thus 550g of a kippi extract were obtained. The kippi extract was dissolved in ethyl acetate in an amount of 5 times the weight of the extract, followed by addition of water in an amount equal to that of the ethyl acetate and stirring, and the mixture was left undisturbed. After separating the mixture into layers, a water layer was removed (washing with water). This operation (washing with water) was repeated three times, and then ethyl acetate was distilled off. Thus, 300g of dry solid product were obtained. Furthermore, this dry solid product was dissolved in a mixed solution of hexane / ethanol (in a volume proportion of 85/15) in an amount of twice the weight of the dry solid product, and thus a dissolved solution was obtained. All the dissolved solution was charged to a column (ϕ 30cm, with a length of 1m) filled with silica gel. Hexane in a volume of twice the volume of the column was allowed to flow in the column, and then, using a mixed solvent of hexane/ethanol (in a volume proportion of 90/10) as an eluent, the elution was fractionated into 1L fractions. When each fraction was analyzed by silica gel thin layer chromatography using a hexane/ethanol mixed solution (volume proportion of 90/10) as a developer, the presence of compounds 1 to 4 was confirmed. The fractions containing these compounds were mixed, and the solvent was distilled off to obtain the compounds 1 to 4. The yields of the compounds were as follows: 7g for compound 1, 63g for compound 2, 33g for compound 3, and 13g for compound 4.

[0041] Each of the obtained compounds 1 to 4 was analyzed by the nuclear magnetic resonance (NMR) spectrum and the mass analysis (MS) spectrum. Figs. 1 to 3 show the ^{1}H-NMR, ^{13}C-NMR and MS spectra of the compound 2, and Figs 4 to 6 show the ^{1}H-NMR, ^{13}C-NMR and MS spectra of the compound 3.

[0042] When these data are compared with the data described in references (Natural Medicines 51(3), 190-193 (1997), Chem. Pharm. Bull. 37, 1092(1989), Pharmacological Magazine (YAKUGAKU ZASSHI) 116(3), 244-250 (1996) and Tetrahedron, 16(8), 64(1964), the compound 2 was identified as 5,6,7,8,3',4'-hexamethoxyflavone (formula (III): nobiletin). The compound 3 was identified as 5,6,7,8,4'-pentamethoxyflavone (formula (IV): tangeretin).

[0043] The structures of the other compounds (compound 1 and compound 4) were determined by comparing their ^{1}H-NMR, ^{13}C-NMR and MS spectra with the ^{1}H-NMR, ^{13}C-NMR and MS spectra of the compounds represented by formulae (III) and (IV). As a result, the compound 1 was identified as 3,5,6,7,8,3',4'-heptamethoxyflavone (formula (II)), and the compound 4 was identified as 5,7,8,3',4'-pentamethoxyflavone (formula (V)).

(Production Example 2: Isolation of polymethoxyflavone)

[0044] First, 10kg of dried peel of Citrus tachibana (herbal name: "kippi") was pulverized with a blender, and immersed for extraction for 5 days in 95% ethanol (first grade) in an amount of 5 times the weight of the dried peel. An extract liquid was filtered and concentrated under reduced pressure, and thus 560g of a kippi extract were obtained. A mixed solvent of hexane/ chloroform (in a volume proportion of 1/1) in an amount of five times the weight was added to the kippi extract, and the mixture was stirred for about 30 min. Thereafter, the mixture was left undisturbed at 4 °C over night, and a supernatant was collected by decantation, followed by distillation of the solvent. Thus, 320g of dry solid product were obtained. This dry solid product was separated and purified by being subjected to column chromatography in the same manner as in Production Example 1, and thus compounds 1 to 4 were collected. The yields of the compounds were as follows: 6g for compound 1, 65g for compound 2, 35g for compound 3, and 14g for compound 4. The results of analysis revealed that the compounds 1 to 4 correspond to the compounds (II) to (V), respectively.

(Confirmation of whitening effect-1: suppression of melanin formation)

[0045] Using the obtained compounds of formulae (II) to (V), the effect of suppressing melanin production of human melanoma cell (HM3KO) was examined in vitro. More specifically, HM3KO was cultured to about 5×10^5 by a conventional method, collected by centrifugation to obtain pellets, and inoculated the pellet to a culture dish of 10cm diameter containing an Eagle's medium supplemented with 10% fetal bovine serum and cultured at 37 °C for 24 hours. Thereafter, each polymethoxyflavone of formulae (II) to (V) was added, or kojic acid or arbutin for comparison was added thereto so that the final addition concentration was 10μM, followed by cultivation for 6 days. After the cultivation, cells were collected by centrifugation, and 1ml of 2N sodium hydroxide aqueous solution was added thereto to obtain cell lysate. The absorbance in a wavelength of 410nm of the cell lysate was measured with a spectrophotometer. Here, the absorbance of the cell lysate to which a sample is not added is defined as 100% of the melanin production ratio, and the melanin production ratios are shown as relative values, measuring the absorbance of each cell lysate. Table 1 shows the results.

Table 1

Sample	Sample concentration in medium (μM)	Melanin production ratio (% of control)
(no addition)	0	100
3,5,6,7,8,3',4'-heptamethoxyflavone : formula (II)	10	65
5,6,7,8,3',4'-hexamethoxyflavone : formula (III)	10	35
5,6,7,8,4'-pentamethoxyflavone : formula (IV)	10	55
5,7,8,3',4'-pentamethoxyflavone : formula (V)	10	60
Kojic acid	10	96
Arbutin	10	97

[0046] The results of Table 1 indicate that all of the polymethoxyflavones of formulae (II) to (V) significantly suppressed melanization of HM3KO. This effects are far beyond those of kojic acid and arbutin.

(Confirmation of whitening effect-2: suppression of UV-induced pigmentation)

[0047] Using brown guinea pigs, the suppression of UV-induced pigmentation was examined in vivo. More specifically, the backs of the brown guinea pigs were shaved and covered with an ultraviolet shielding plate provided with 6 rectangular holes of 2 × 2cm, followed by irradiation of UV ($0.5J/cm^2$) to induce pigmentation. Thereafter, 40 μL of 70% (W/W) ethanol aqueous solution containing each of polymethoxyflavone (nobiletin) of formula (III) or polymethoxyflavone (tangeretin) of formula (IV) in a concentration shown in Table 2 were applied twice a day. Then, the degrees of pigmentation before and after the application were measured. Table 2 shows the results.

Table 2

Sample	Sample concentration (%)	Pigmentation degree ΔL		
		Before irradiation	After 20 days	After 40 days
(no addition)	0	0	-7.8	-5.2
5,6,7,8,3',4'-hexamethoxyflavone: formula (III)	4	0	-5.1	-1.9
5,6,7,8,3',4'-hexamethoxyflavone: formula (III)	0.4	0	-5.5	-3.2
5,6,7,8,4'-pentamethoxyflavone: formula (IV)	4	0	-5.6	-3.3
5,6,7,8,4'-pentamethoxyflavone: formula (IV)	0.4	0	-6.1	-3.5

[0048] In Table 2, the pigmentation degree Δ L was obtained by measuring a L value before UV irradiation, which is defined as 0, with a spectrophotometric colorimeter, and obtaining the difference between the L values before and after UV irradiation. A lower value ΔL corresponds to a higher pigmentation degree.

[0049] Table 2 indicates that the polymethoxyflavone used in the present invention significantly suppress the induction of pigmentation of brown guinea pigs.

Examples 1 to 4 and Comparative Examples 1 to 3

[0050] Cosmetic compositions for skin lotion containing components shown in Table 4 were prepared by using five components shown in Table 3 as common components. The polymethoxyflavones used were the polymethoxyflavones of formulae (III) and (IV) that were isolated and purified in the production examples.

Table 3

Commonly added components		
	Components	Added amount (wt%)
1	Trisodium citrate dihydrate	0.3
2	Ethanol	3
3	Methylparaben	0.1
4	Phenoxyethanol	0.2
5	Sodium sulfate anhydride	0.1
Total		3.7

[0051] The obtained cosmetic compositions were evaluated by the following method. Table 4 shows the results.

(1) Whitening effect

[0052] Evaluation was carried out with 50 women (in their 20's and 30's) as test persons, and the cosmetic compositions were used twice a day, that is, in the morning and at night, for 2 months. Then, the improvement degree in "liver spots" and "freckles" after use was determined by eyesight with the following criteria.

[0053] 10 points: when it was determined that the composition was evidently effective.

[0054] 5 points: when it was determined that the composition was slightly effective.

[0055] 0 point: when it was determined that the composition was not effective at all.

[0056] The average of the points of the 50 women was obtained, and a cosmetic composition for which the average

was 5 points or more was defined as a cosmetic composition having high whitening effect.

G: high whitening effect (5 points or more in average)
P: low whitening effect (below 5 points in average)

(2) Moisture of the skin

[0057] Evaluation was carried out with 20 women (of the age between 22 and 32) as test persons, and the cosmetic compositions were used after washing their faces. Then, moisture of the skin immediately after use and 2 hours later were determined with the following criteria.
[0058] 2 points: when it was perceived that the moisture of the skin was sufficient immediately after use, and the skin was still moist 2 hours later.
[0059] 1 point: when it was perceived that the moisture of the skin was slightly not sufficient immediately after use, or the skin was slightly dry 2 hours later.
[0060] 0 point: when it was perceived that the moisture of the skin was not sufficient.
[0061] The average of the points of the 20 women was obtained, and a cosmetic composition for which the average was 1.5 points or more was defined as a cosmetic composition having a high effect of allowing the skin to retain moisture.

G: high effect of allowing the skin to retain moisture (1.5 points or more in average)
P: low effect of allowing the skin to retain moisture (below 1.5 points in average)

(3) Vitality of the skin

[0062] Evaluation was carried out with 20 women (of the age between 22 and 32) as test persons, and the cosmetic compositions were used after washing their faces. Then, the vitality of the skin was determined with the following criteria.
[0063] 2 points: when it was perceived that the skin was vitalized.
[0064] 1 point: when it was perceived that the skin was slightly vitalized.
[0065] 0 point: when it was perceived that the skin was not vitalized.
[0066] The average of the points of the 20 women was obtained, and a cosmetic composition for which the average was 1.5 points or more was defined as a cosmetic composition having a high effect of vitalizing the skin.

G: high effect of vitalizing the skin (1.5 points or more in average)
P: low effect of vitalizing the skin (below 1.5 points in average)

(4) Wrinkle suppressing effect

[0067] Evaluation was carried out with 20 women (of the age between 24 and 35) as test persons, and the cosmetic compositions were used twice a day (in the morning and at night) for two weeks in a row. Then, the wrinkle suppressing effect was determined with the following criteria.
[0068] 2 points: when it was perceived apparently that wrinkles were not conspicuous any more.
[0069] 1 point: when it was perceived that wrinkles were slightly not conspicuous.
[0070] 0 point: when it was perceived that there was no wrinkle suppressing effect.
[0071] The average of the points of the 20 women was obtained, and a cosmetic composition for which the average was 1.5 points or more was defined as a cosmetic composition having a high effect of suppressing wrinkles.

G: high effect of suppressing wrinkles (1.5 points or more in average)
P: low effect of suppressing wrinkles (below 1.5 points in average)

(5) Storage stability

[0072] The cosmetic compositions were sealed in transparent glass containers, and stored at 0°C, 25°C, and 40°C for three months. The appearance thereof was observed, and evaluation was carried out with the following criteria.

G: Good stability (there is no change in the appearance at any temperatures)
S: Slightly poor stability (A sediment or precipitate slightly occurs, or slight coloring occurs at some temperatures)
P: Poor stability (A sediment or precipitate occurs or separation occurs at some temperatures, or coloring is significant.)

[0073] Table 4 shows the results.

Table 4

	Examples (wt%)				Comparative Examples (wt%)		
	1	2	3	4	1	2	3
a 5,6,7,8,3',4'-hexamethoxyflavone: formula (III)	2	---	0.5	0.3	---	---	---
a. 5,6,7,8,4'-pentamethoxyflavone: formula (IV)	---	2	---	0.1	---	---	---
Placental extract	---	---	---	---	---	0.5	---
Ascorbic acid magnesium phosphate salt	---	---	0.5	---	---	0.5	3
Glycerol	---	2	2	2	2	2	2
Dipropylene glycol	---	3	3	3	3	3	3
Citrate monohydrate	0.1	0.1	---	0.1	0.1	---	--
Commonly added components	3.7						
Purified water	the rest						
Total	100						
(1) Whitening effect	G (7.0)	G (6.3)	G (7.5)	G (7.2)	P (1.8)	P (2.2)	P (3.7)
(2) Skin moisture	G (1.7)	G (1.8)	G (1.9)	G (1.8)	P (1.2)	P (1.4)	P (1.4)
(3) Skin vitality	G (1.7)	G (1.9)	G (1.8)	G (1.7)	P (1.3)	P (1.3)	P (1.3)
(4) Wrinkle suppressing effect	G (1.8)	G (1.9)	G (1.8)	G (1.7)	P (1.2)	P (1.2)	P (1.3)
(5) Storage stability	G	G	G	G	G	G	P

[0074] According to Examples 1 to 4, the lotions containing polymethoxyflavones of the present invention are excellent in the whitening effect, allow the skin to retain moisture for a long time, and vitalize the skin, and suppress wrinkles, and have excellent storage stability. On the other hand, Comparative Examples 1 to 3 cannot provide cosmetic compositions with adequate performance. More specifically, in Comparative Example 1, since no polymethoxyflavone is contained, almost no whitening effect is provided. In Comparative Example 2 where placental extract is used instead of the polymethoxyflavone, the whitening effect is weak, and there is no effect on the skin moisturization, the skin vitalization, and the suppression of wrinkles. In Comparative Example 3 where ascorbic acid salt is used instead of the polymethoxyflavone, the whitening effect is weak, and there is no effect on the skin moisturization, the skin vitalization, and the suppression of wrinkles, and the storage stability is also problematic.

Examples 5 to 7

[0075] Cosmetic whitening compositions for oil-in-water milky lotion shown in Table 8 were prepared by using the 12 components shown in Table 5 as common components.

Table 5

Commonly added components		
	Components	Added amount (wt%)
1	Xanthan gum (manufactured by Dainippon Pharmaceutical Co., Ltd., "ECHO GUM T")	0.1
2	Carboxyvinyl polymer (BFGoodrich Co., Ltd., "carbopol 940")	0.12
3	Ethanol	5
4	Purified sunflower oil	5

Table 5 (continued)

Commonly added components		
	Components	Added amount (wt%)
5	Squalane	3
6	Octyldodecyl myristate	2
7	Polyoxyethylene(32mol)monostearate	0.5
8	Polyoxyethylene (20mol) stearyl ether	0.7
9	Glycerol monostearate	1
10	Methylparaben	0.1
11	Butylparaben	0.05
12	Phenoxyethanol	0.2
Total		17.77

[0076] The obtained milky lotions were evaluated in the same manner as in Examples 1 to 4. Table 8 shows the results.

Examples 8 and 9

[0077] Cosmetic skin care compositions for oil-in-water creams shown in Table 8 were prepared by using the 12 components shown in Table 6 as common components.

Table 6

Commonly added components		
	Components	Added amount (wt%)
1	Purified olive oil	8
2	Squalane	3
3	Octyldodecyl myristate	2
4	Dimethylpolysiloxane (100CS)	1
5	Purified yellow wax	3
6	Polyoxyethylene (20mol) sorbitan monostearate	1
7	Polyoxyethylene(75mol)monostearate	1
8	Glycerol monostearate	2
9	Tocopherol acetate	0.05
10	Methylparaben	0.2
11	Propylparaben	0.1
12	Butylparaben	0.1
Total		21.45

[0078] The obtained oil in water creams were evaluated in the same manner as in Examples 1 to 4. The storage stability was evaluated with the method shown in (5') below.

(5') Storage stability

[0079] The cosmetic compositions were sealed in transparent glass containers, and stored at - 5°C, 25°C, and 45°C for one month. Then, their state was examined, and evaluation was carried out with the following three criteria.

G: Good stability (there is no change in the appearance at any temperatures, and there is no aggregation or the like)
S: Slightly poor stability (slight precipitation occurs, or slight separation occurs at some temperatures, or aggregations or lumps are formed slightly.)
P: Poor stability (Apparently, a precipitate occurs or separation occurs at some temperatures, or aggregations or lumps appear.)

[0080] Table 8 shows the results.

Examples 10 and 11

[0081] Cosmetic skin care compositions for water-in-oil creams shown in Table 8 were prepared by using the 12 components shown in Table 7 as common components.

Table 7

Commonly added components		
	Components	Added amount (wt%)
1	Purified jojoba oil	3
2	Purified sunflower oil	6
3	Dimethylpolysiloxane (100CS)	1
4	Squalane	4
5	Octyldodecyl myristate	5
6	Glycerol monooleate	1.5
7	Diglycerol monooleate	0.2
8	Tocopherol acetate	0.1
9	Methylparaben	0.2
10	Propylparaben	0.1
11	Butylparaben	0.1
12	Magnesium sulfate (heptahydrate)	0.5
Total		21.7

[0082] The obtained water-in-oil creams were evaluated in the same manner as in Examples 8 and 9.
[0083] Table 8 shows the results.

Table 8

	Examples (wt%)						
	o/w milky lotion			o/w cream		w/o cream	
	5	6	7	8	9	10	11
a5,6,7,8,3',4'-hexamethoxyflavone: formula(III)	1	-	0.5	2	0.5	1	2
a.5,6,7,8,4'-pentamethoxyflavone: formula(IV)	-	2	-	-	1	-	1
Glycerol	2	2	2	2	2	2	2
Dipropylene glycol	2	2	2	3	3	3	3
Polyethylene glycol 1540	2	2	2	-	-	-	-
Ascorbic acid magnesium phosphate salt	-	-	-	0.5	-	-	-
L-arginine	0.1	0.1	0.1	-	-	-	-

Table 8 (continued)

	Examples (wt%)						
	o/w milky lotion			o/w cream		w/o cream	
	5	6	7	8	9	10	11
Cetanol	2	-	-	3	-	-	3
Behenyl alcohol	-	1	1	-	3	2	-
Decamethylcyclosiloxane	-	-	-	3	-	-	-
Commonly added components	17.77(table 5)			21.45(table 6)		21.7(table 7)	
Purified water	the rest						
Total	100						
(1) Whitening effect	G (7.1)	G (6.4)	G (7.8)	G (7.2)	G (6.8)	G (7.6)	G (8.1)
(2) Skin moisture	G (1.9)	G (1.9)	G (1.8)	G (1.9)	G (1.9)	G (2.0)	G (2.0)
(3) Skin vitality	G (1.8)	G (1.9)	G (1.9)	G (1.8)	G (1.9)	G (1.8)	G (1.8)
(4) Wrinkle suppressing effect	G (1.9)	G (2.0)	G (1.9)	G (1.9)	G (1.9)	G (1.9)	G (1.8)
(5) Storage stability	G	G	G	-	-	-	-
(5')Storage stability	-	-	-	G	G	G	G

[0084] According to Examples 5 to 7, all the cosmetic compositions (milky lotions) of the present invention have an excellent whitening effect, allow the skin to retain moisture for a long time, vitalize the skin, suppress wrinkles, and have excellent storage stability.

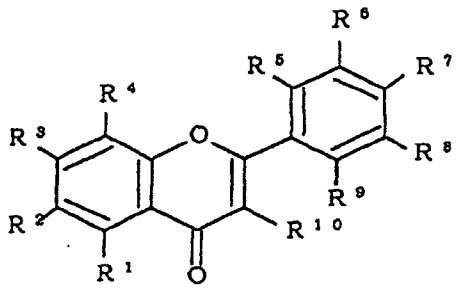
[0085] According to Examples 8 and 9, all the cosmetic skin care compositions (creams) of the present invention have an excellent whitening effect, allow the skin to retain moisture for a long time, vitalize the skin, suppress wrinkles, and have excellent storage stability.

[0086] According to Examples 10 and 11, all the cosmetic compositions (creams) of the present invention have an excellent whitening effect, allow the skin to retain moisture for a long time, vitalize the skin, suppress wrinkles, and have excellent storage stability.

[0087] The invention may be embodied in other forms without departing from the spirit or essential characteristics thereof. The embodiments disclosed in this application are to be considered in all respects as illustrative and not limiting. The scope of the invention is indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are intended to be embraced therein.

Claims

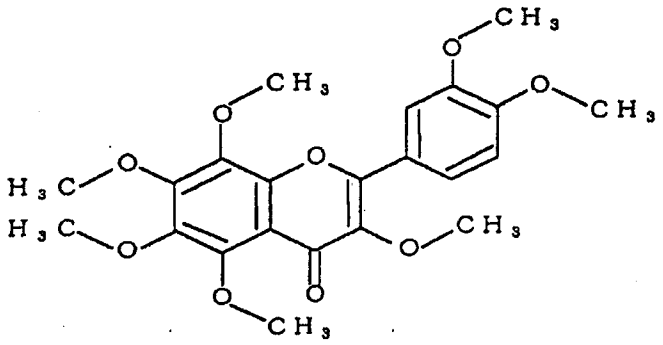
1. A cosmetic composition comprising 0.00005 to 10wt% of polymethoxyflavone represented by formula (I):



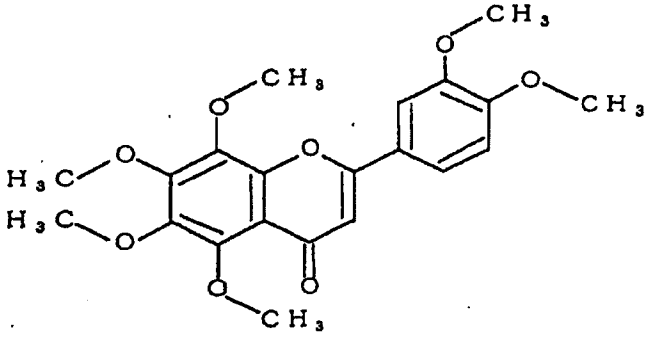
(I)

wherein each of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} is selected from the group consisting of hydrogen atom, hydroxyl group, alkoxy group having 1 to 20 carbon atoms, alkyl group having 1 to 20 carbon atoms, alkenyl group having 2 to 20 carbon atoms, hydroxyalkyl group having 1 to 20 carbon atoms or a sugar residue, and at least four of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are methoxy groups.

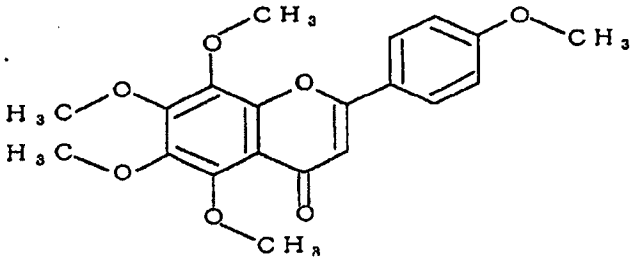
2. The cosmetic composition of claim 1, wherein the polymethoxyflavone comprises at least one compound selected from the group consisting of compounds represented by formulae (II) to (V):



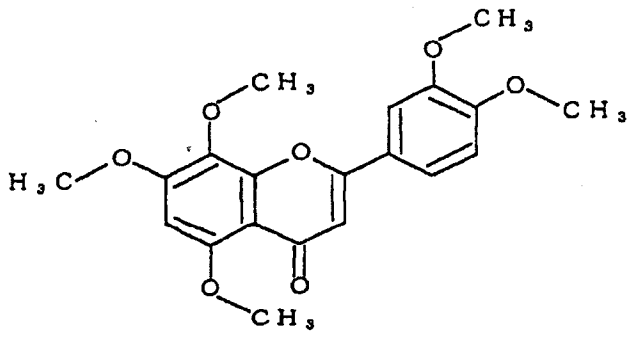
(II)



(III)



(IV)



(V)

3. The cosmetic composition of claim 2, wherein the polymethoxyflavone comprises at least one compound selected from the group consisting of 5,6,7,8,3',4'-hexamethoxyflavone and 5,6,7,8,4'-pentamethoxyflavone.

4. A method for isolating and purifying polymethoxyflavone comprising the steps of:

. subjecting peel of a plant of the Genus Citrus of the Family Rutaceae to extraction with at least one solvent selected from the group consisting of methanol, ethanol, propanol, butanol, ethyl acetate, acetone, propylene glycol, and 1, 3-butylene glycol to obtain an extract (S1);
dissolving the extract (S1) in ethyl acetate, adding water thereto, stirring, separating into layers, removing a water layer, distilling off the ethyl acetate to obtain a dry solid product (S2); and
dissolving the dry solid product (S2) in a solvent, and subjecting it to liquid column chromatography.

5. A method for isolating and purifying polymethoxyflavone comprising the steps of:

subjecting peel of a plant of the Genus Citrus of the Family Rutaceae to extraction with at least one solvent selected from the group consisting of methanol, ethanol, propanol, butanol, ethyl acetate, acetone, propylene glycol, and 1, 3-butylene glycol to obtain an extract (S1);
dissolving the extract (S1) in hexane and/or chloroform, removing a precipitate, distilling off the hexane and/or chloroform to obtain a dry solid product (S3); and
dissolving the dry solid product (S3) in a solvent, and subjecting it to liquid column chromatography.

6. The method of claim 4 or 5, wherein the liquid column chromatography uses silica gel and/or alumina as a filler, and a mixed solution of hexane/ethanol in a volume proportion of 70/30 to 97/3 as an eluent.

Fig. 1

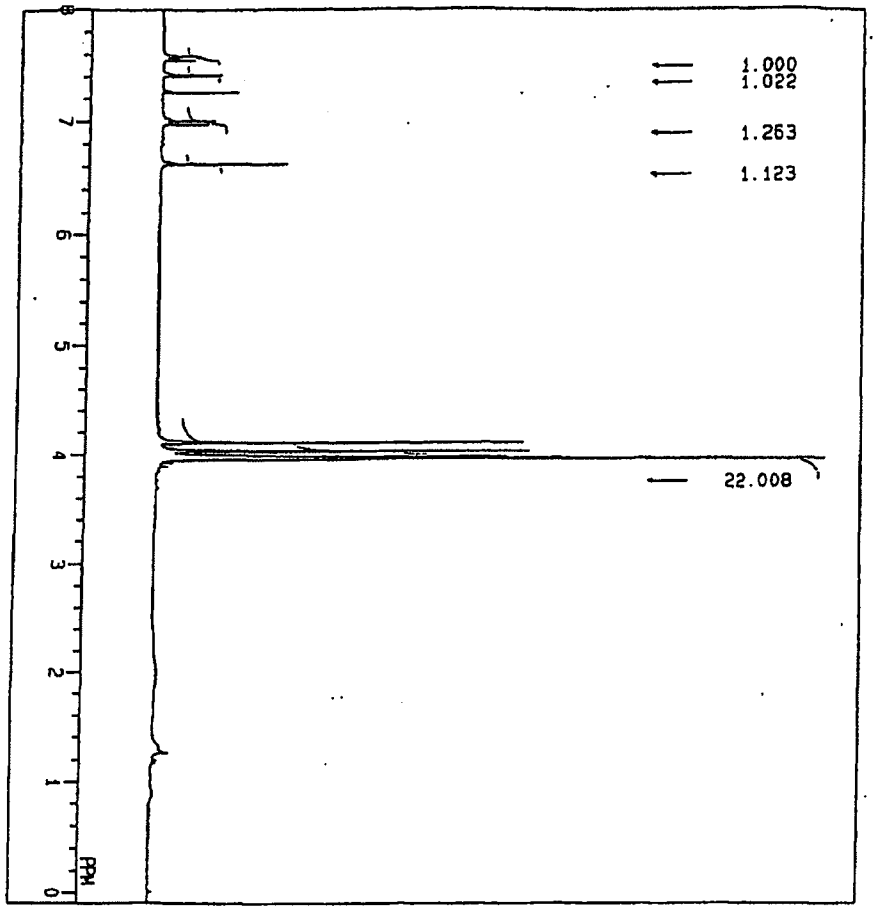


Fig. 2

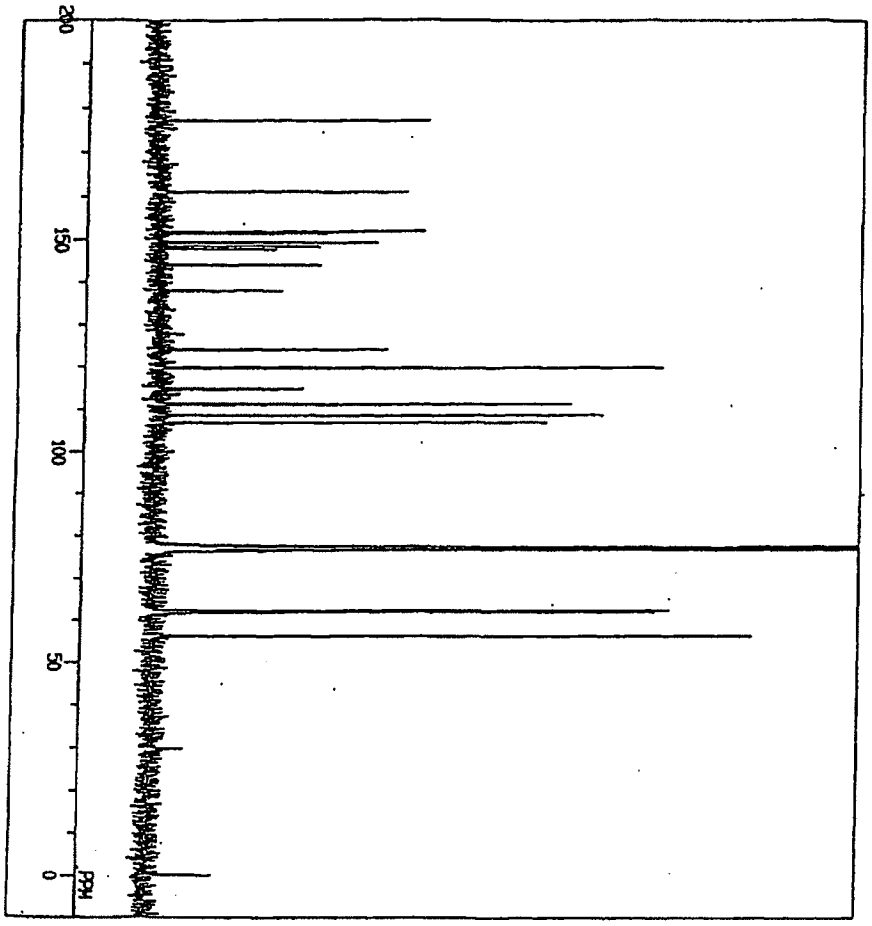


Fig. 3

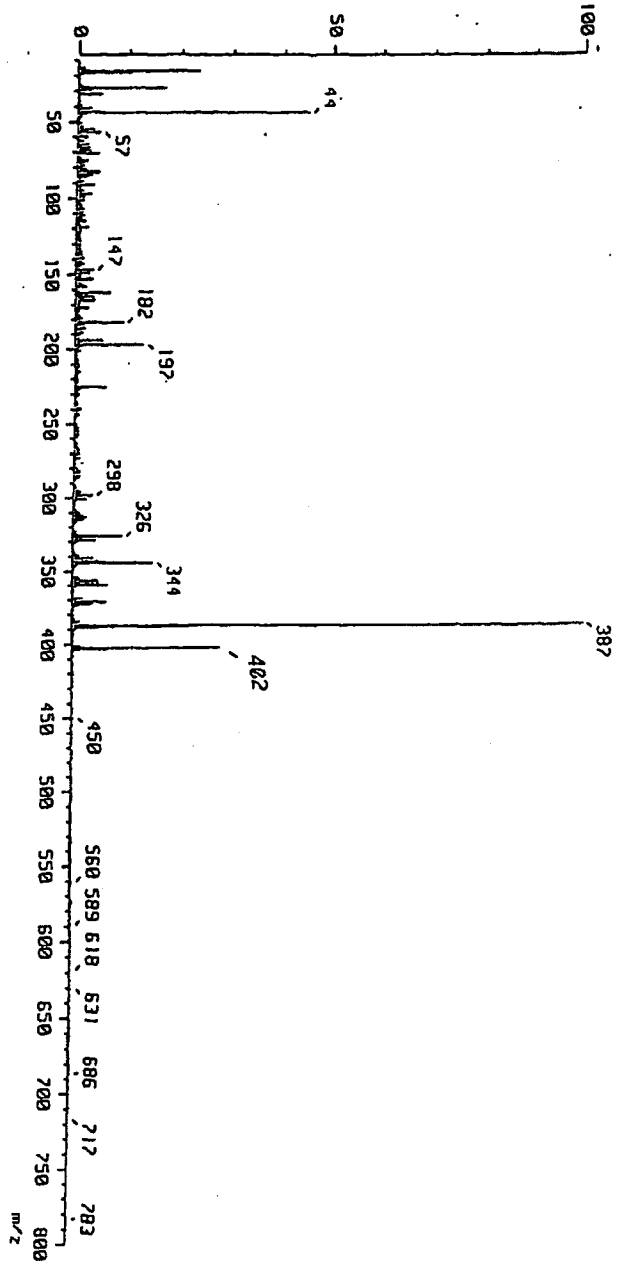

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Fig. 4

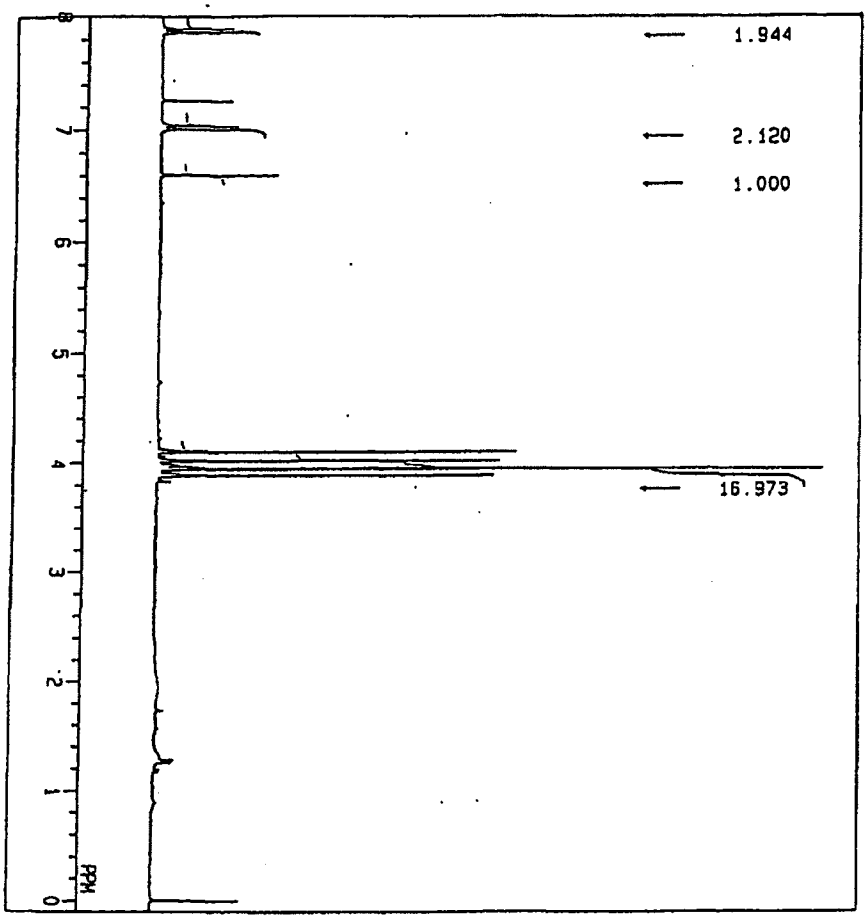


Fig. 5

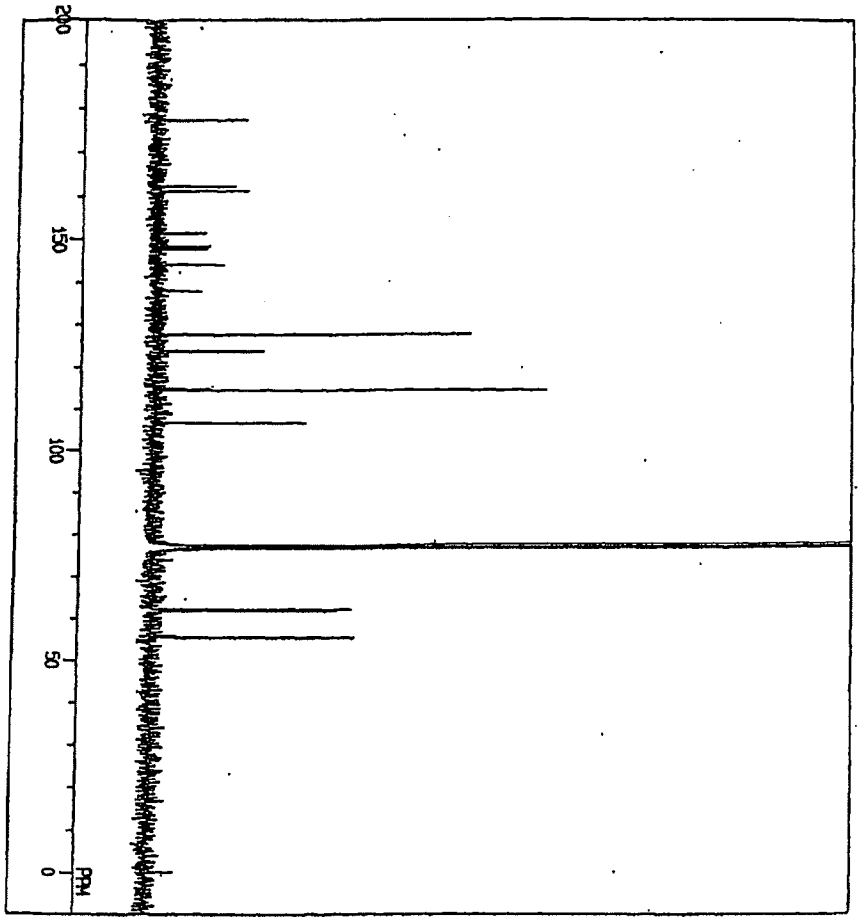
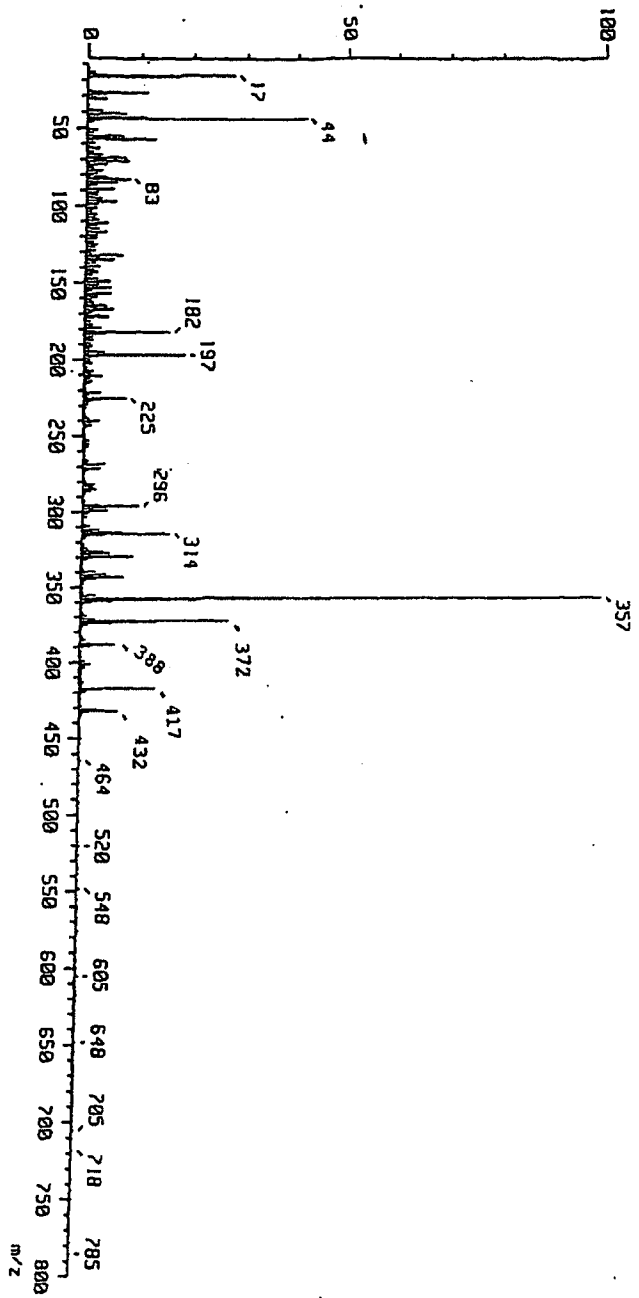


Fig. 6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